D-site Binding Protein Transactivation Requires the Proline- and Acid-rich Domain and Involves the Coactivator p300*

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The D-site binding protein (DBP) is a member of the proline- and acid-rich (PAR) domain subfamily of basic/leucine zipper proteins and is involved in transcriptional regulation in the liver. Deletion analysis of the DBP protein was carried out in an effort to define the function of the conserved PAR domain. Internal deletions of the protein, i.e. removing portions of the PAR domain, resulted in a substantial loss in transactivation of a high affinity DBP reporter construct when assayed in Hep G2 cells. These same sequences conferred significant transactivation to GAL4 DNA binding domain fusion proteins, indicating that this region acts as part of an independent activation domain comprised of sequences in both the amino terminus and in the PAR domain of DBP. The coexpression of full-length expression constructs for both DBP and hepatic leukemia factor resulted in a dramatic increase in activation mediated by the GAL4-DBP fusion proteins, suggesting the involvement of a regulated coactivator in this process. DBP transactivation appears to be a p300-dependent process, as a 12 S E1A expression construct disrupted DBP-mediated transactivation, and a p300 expression vector, but not a CREB binding protein vector, was able to restore DBP transactivation. These results suggest that the PAR domain is required for DBP activation, which occurs through a regulated, p300-dependent process.

The D-site binding protein (DBP)* is a member of the basic/leucine zipper family (b/ZIP) and was first isolated by its ability to bind and transactivate through an element in the serum albumin promoter (1). DBP mRNA is detected in most tissues, although the protein accumulates to high levels only in the nuclei of adult liver, suggesting that it is posttranscriptionally regulated. This 43-kDa protein is also known to undergo a basic extension, that is thought to be important for DNA binding specificity (10).

In this study we have been able to define a role for the PAR domain of DBP in the transactivation potential of this protein. Through the use of deletion mutants within the context of the whole DBP protein, we have demonstrated that sequences within the central 28 amino acids of the PAR domain are essential for DBP to transactivate a site from the C7aH gene (C7aH), which can be transactivated by DBP and which also exhibits a significant diurnal regulation (3). Other genes that appear to be regulated by DBP include PEPCK (4), alcohol dehydrogenase (5), carbamyl phosphate synthetase (6), and P_{450} CYP2C6 (7). DBP may also be involved in the regulation of the clotting factor IX gene and recovery of the Leyden phenotype form of hemophilia B (8, 9).

In addition to the b/ZIP domain, DBP also contains a distinct region termed the proline- and acid-rich (PAR) domain, which consists of a large number (16%) of proline, glutamate, and aspartate residues. This unique region, found immediately amino-terminal to the basic domain, shares 83% identity with another b/ZIP transcriptional regulators, thyrotrropic embryonic factor (TEF) (10) and hepatic leukemia factor (HLF) (11, 12), and together they form the subfamily of PAR proteins. TEF is expressed in the rat anterior pituitary gland and is one of the key factors in transactivating the thyroid-stimulating hormone β promoter (10). HLF was discovered through studies of the human pre-B acute lymphoblastic leukemia bearing 17:19 chromosomal translocations where the b/ZIP domain of HLF was found fused to the E2A gene (11, 12). HLF expression has been detected in the liver, though little is known about the roles of TEF and HLF in this tissue. All three members of the PAR subfamily recognize related sequences and are able to heterodimerize (10, 13, 14). Despite the highly conserved nature of the PAR domain, no unique function has been associated with it. Mutational analysis of the TEF PAR domain identified a cluster of six basic amino acids, referred to as the “basic extension,” that is thought to be important for DNA binding specificity (10).

In this study we have been able to define a role for the PAR domain of DBP in the transactivation potential of this protein. Through the use of deletion mutants within the context of the whole DBP protein, we have demonstrated that sequences within the central 28 amino acids of the PAR domain are essential for DBP to transactivate a site from the C7aH promoter. Using GAL4 DNA binding domain fusion proteins, we have shown that the PAR domain must act in conjunction with sequences within the amino terminus of DBP to mediate transactivation. Interestingly, cotransfection of full-length DBP or HLF was able to dramatically increase the transactivation potential of these GAL4-DBP fusion constructs. Cotransfection of an E1A vector was able to inhibit DBP-mediated transactivation. Introduction of a p300 expression vector was able to overcome the E1A-mediated inhibition. These results indicate that DBP transactivation is a p300-dependent process.

MATERIALS AND METHODS

Nuclear Extracts and DNA Binding Assays—Nuclear extracts were prepared from Hep G2 cells transfected with calcium phosphate coprecipitation with DBP and DBP/GAL4 expression vectors. After 48 h, three 100-mm tissue culture dishes transfected with each construct were washed, and the cells were collected and spun at 1000 × g for 5 min. The pellet was resuspended in 4 ml of lysis buffer (10 mM Hepes,
pH 7.6, 100 mM KCl, 0.1 mM EDTA, 3 mM MgCl₂, 10% glycerol, 1 mM dithiothreitol, 1% Trasylol, and 1 μg/ml leupetin/pepsatin A) and ruptured with 10 strokes of a Dounce homogenizer. The mixture was spun at 500 × g for 5 min, and the pellet was resuspended in 1 ml of the above lysis buffer. Cells were again pelleted for 2 min at 15,000 × g in a microfuge, and the protein fraction was isolated by resuspension in 150 μl of lysis buffer with 0.27 mM KCl. This mixture sat on ice for 15 min with intermittent mixing, was spun for 15 min at 15,000 × g, and the protein-containing supernatant was stored at −70 °C.

Gel mobility assays were performed as described previously (8). The sequences of the oligonucleotides used in these experiments are as follows.

D-site 5′-TGGTGATTTTGAGGGCTGTGGCTCTGACAGGAGGCTTCGA-3′
A site 5′-CTTGATTTTGAGGGCTGTGGCTCTGACAGGAGGCTTCGA-3′
C7αH 5′-CTTGGATTTTGAGGGCTGTGGCTCTGACAGGAGGCTTCGA-3′
17–G4M 5′-AGCTTGGAGGCTGTGGCTCTCGGCTCCTGACAGGAGGCTTCGA-3′

Oligonucleotides 1–3 for bandshift probes

Supershift experiments were carried out by the preincubation of the antibody with the protein extracts in the bandshift binding reaction for 30 min on ice. The sequence of the radiolabeled oligonucleotide was then cloned into the G-free/TATA/LUC vector or the GAL4 DNA binding domain. Constructs, including the NH₂-terminal portion of DBP (amino acids 1–185) upstream of the TATA box, were created by insertion of a 17–G4M construct at the H1 fragment encoding the PAR domain alone were cloned into the vector G4MpolyII. Vectors contained the PAR domain (amino acids 1–148) in the vector G4MpolyII. Vectors containing the PAR domain deletions was not the result of changes in intrinsic transactivation and that a core region of close to 30 amino acids of deletions). These constructs preserved the amino-terminal half of DBP as well as the bZIP domain so that changes in DNA binding specificity were expected to be minimal. Previous studies have demonstrated the ability of DBP to transactivate via the high affinity D-site D-site in Hep G2 cells. In this work we have taken advantage of a reporter construct consisting of multimerized DBP binding elements derived from the C7αH promoter (3) cloned in front of the albumin TATA box and driving a G-free cassette as well as the luciferase gene. This vector has proven to be more specific for DBP than the albumin D-site element, as transactivation due to the presence of CCAAT/enhancer binding protein α in these cells is considerably reduced with this construct. Expression of the full-length DBP construct results in an over 250-fold increase in promoter activity (Fig. 1B) compared with the empty expression vector (pSCT). Deletion of the first 20 amino acids (PAR2) resulted in little change in transactivation. However, progressive deletions through the next 27 amino acids resulted in a steady loss of activity. The PAR3 construct was reduced to a 30-fold activation, whereas the BXT construct (which eliminates the PAR domain completely) was essentially inactive. These results indicate that the PAR domain is critical for DBP transactivation and that a core region of close to 30 amino acids within this region is of particular importance.

To confirm that the decreased transactivation mediated by the PAR domain deletions was not the result of changes in protein stability or DNA binding, nuclear extracts of Hep G2 cells transfected with the various constructs were prepared. Western blot analysis indicated that all of the constructs produced comparable amounts of protein with the expected mobility (data not shown). The extracts were then normalized for equal amounts of DBP protein and were used in gel mobility shift assays, with the DBP binding elements from the C7αH gene promoter (–237 to –212) (3) and the D-site of the albumin promoter (1) as probes. Two different probes were used to determine whether these mutations altered binding specificity. All of the PAR deletions appear to have a slight increase in DNA binding activity compared with the wild type sequence. Little or no decrease in relative DNA binding activity was observed with either probe until a region known as the basic extension was removed (Fig. 1C, BXT), which resulted in complete loss of DNA binding activity. This is consistent with the reported involvement of this region in modulating DNA binding specificity in TEF. In contrast to the other deletion mutants, the DNA-protein complex formed with the PAR3 construct was characterized diffuse. This change in migration may be associated with conformational changes formed with the PAR3 construct, which results in partial binding of associated proteins. Overall, these results indicate that the observed decrease in DBP-mediated transactivation upon removal of parts of the PAR domain (through to PAR3), is the result of changes in intrinsic transactivation activity and not the result of reduced DNA binding activity. Clearly, removal of the basic extension does have a dramatic effect on DNA binding, resulting in complete loss of transactivation.

Transactivation mediated by DBP has previously been shown to be tissue-specific, being active in Hep G2 cells, which are derived from a hepatocellular carcinoma but inactive in L
To determine whether the PAR domain plays a role in this tissue-specific activation, the cotransfection experiments with the PAR deletions were performed in CV-1 cells, a monkey kidney cell line. No transactivation was observed in this cell line even with the deletions (data not shown), indicating that the PAR domain does not play a negative role in the generation of tissue specificity. As DBP only transactivates in Hep G2 cells, it is possible that other factors that are liver-specific may be required for DBP transactivation.

**The PAR Domain Is Part of the Transactivation Domain**—To determine whether the PAR domain functions independently as a transactivation domain, fusion constructs with the GAL4 DNA binding domain were created. Each of the PAR deletion constructs, in conjunction with the entire NH$_{2}$-terminal portion of DBP (amino acids 1–185), was fused upstream of a 147-amino acid region of GAL4, encoding its DNA binding domain in an SV40-based expression vector. The GAL4-responsive reporter construct consisted of two repeats of the GAL4 binding site cloned into the G-free/TATA/LUC reporter construct (see Fig. 3A). Transfection with a construct containing the entire amino-terminal and PAR domains of DBP into Hep G2 cells resulted in a 24-fold increase in activity (Fig. 2A, GNP) compared with transfection of the GAL4 DNA binding domain alone (G4M). Progressive deletion of the PAR domain in the presence of the amino terminus resulted in a decrease in transcriptional activity similar to that seen with the whole protein (Fig. 2A: GNP, 11-fold; GNP1, 11-fold; GNP2, 4-fold; and GNP3, 4-fold). This effect of the PAR domain was dependent on sequences within the amino terminus, as the PAR domain alone (GP, 0.9-fold) exhibits no activity.

To address whether the deletions alter protein stability or DNA binding activity, nuclear extracts from Hep G2 cells transfected with each of the GAL4-DBP deletion constructs were prepared. Western blot analysis using a DBP antibody indicated that all of the fusion proteins produced comparable amounts of protein (data not shown). Bandshift assays with
these extracts were also carried out using the GAL4 recognition site. The complexes formed with the GAL4 fusion containing only the PAR domain (Fig. 2B, GP) or the GAL4 DNA binding domain alone (G4M) were distinct. However, the other N-terminal-containing proteins formed relatively diffuse complexes. A supershift experiment with a DBP antibody was used to specifically identify the fusion protein binding component (Fig. 2B, +Ab). All of the DBP-containing proteins produced a comparable amount of supershifted complex (SS) (slightly less GNP2.2 was added in this experiment). Overall, these results suggest that the differences in transactivation mediated by the GAL4 fusions are likely because of an intrinsic activity of these proteins rather than changes in the amount or DNA binding activity of the proteins produced.

The PAR Proteins Modulate DBP Transactivation—Deletion of the PAR domain in the context of the GAL4 constructs produced an effect very similar to that observed with the whole protein. However, comparison of the level of transactivation obtained with the GAL4 fusions with that of the whole DBP protein or with GAL4 constructs containing the VP16 activation domain revealed that the activity of the GAL4-DBP fusion construct was relatively low. The only domain not included in the GAL4 constructs was the b/ZIP domain, which is involved in DNA binding and dimerization. This disparity in activity suggests that either dimerization was required for optimal transactivation or that the presence of a functional protein increased transactivation. The GAL4 transactivation experiments were repeated in the presence of expression vectors for full-length DBP and HLF. DBP and HLF together produced the most dramatic increase in transactivation, with over a 10-fold increase in activity (Fig. 3B). This activity was dependent on the presence of both the amino-terminal domain and the PAR domain, as constructs containing either domain alone (Fig. 3A, GPN and GNP3) exhibited little stimulation in the presence of the PAR proteins (Fig. 3B).
Interestingly, CCAAT/enhancer binding protein α, which recognizes many of the same sites as DBP, was unable to activate the GAL4-DBP fusion construct (data not shown). The absence of the bZIP domain in the GAL4 fusion proteins should prevent interaction between the fusion protein and the full-length PAR proteins. Attempts to demonstrate physical interaction, either in vivo or in vitro, have failed. These results suggest that the full-length PAR proteins in some way modulate the activity of cellular components involved in transcriptional activation. The requirement of this modulated factor for optimal transactivation mediated by DBP suggests that this factor has the properties of a coactivator.

**DBP Transactivation Is a p300-dependent Process**—The adenoviral early protein E1A has been shown to inhibit specific gene expression, as well as the induction of differentiation, in several model systems by inhibiting the coactivators p300 and CBP (15). To determine whether these proteins play a role in DBP-mediated transactivation, a 12 S E1A expression vector was cotransfected along with the wild type DBP vector and the multimerized C7aH-site reporter construct. Binding of DBP to its response element in this experiment resulted in a 250-fold increase in activity (Fig. 4A). The coexpression of E1A reduced this activity by a factor of 10, suggesting that a protein bound and inactivated by E1A was involved in this process. To directly test whether it is p300 or CBP that is required for DBP activity, complementation of the E1A inhibition was attempted using expression vectors for both proteins. Increasing concentrations of a p300 expression vector were introduced along with the E1A-containing vector. Relatively low concentrations of the p300 expression vector were able to completely restore DBP transactivation (Fig. 4A), though this effect decreased at higher p300 levels. The effect of introducing p300 alone, without E1A, was also examined. At the highest concentration of p300 vector used, DBP-mediated transactivation was reduced to approximately 10% (Fig. 4A), the same level as observed with the E1A expression vector. The tendency of p300 to form inactive aggregates when cotransfected may sequester the endogenous p300, as has been observed previously (16), resulting in the reduced activity observed. In the presence of E1A, overall p300 levels must be stabilized, allowing for complementation. Cotransfection of an expression vector for CBP, which can also bind E1A, did not have any effect on DBP transactivation or on the inhibition of transactivation by E1A (Fig. 4B). Even though both p300 and CBP bind E1A, it is possible that p300 simply sequesters E1A, preventing it from inhibiting some other cellular factor. A p300 mutant that lacks the E1A binding domain (p300Δ30) but is still functional (16) was used in the same complementation assay. It resulted in comparable complementation to the wild type p300 construct (Fig. 4C). Interestingly, expression of the p300Δ30 construct alone did not inhibit transactivation. This may be the result of a reduced tendency of this p300 mutant to aggregate (16). Overall, these experiments demonstrate that DBP transactivation requires a coactivator that can be inhibited by the adenoviral E1A protein and that this coactivator is likely p300.

**DISCUSSION**

The members of the PAR domain family of proteins are unique among the bZIP class of factors in having an extended region of homology outside of the bZIP domain itself. Previous studies have implicated a relatively restricted part of the PAR domain, called the basic extension, in site-specific recognition (10). However, a role for the relatively large remaining portion of this domain has not previously been defined. We have been able to demonstrate a requirement for this domain in transactivation mediated by DBP. Deletions that remove amino-terminal parts of the PAR domain but that do not affect DNA binding activity are severely debilitated in their ability to activate transcription via a high affinity DBP binding site. These same sequences are required for activity of the GAL4 fusion proteins, and again this region functions in conjunction with other sequences present in the amino-terminal part of DBP, as either domain alone is inactive. The amino-terminal domains of all three PAR proteins have a 28-amino acid region of high similarity that may comprise the functional part of the amino-terminal region of DBP (17). It has been shown that this isolated segment from TEF acts as a transcriptional activator when assayed outside the context of the whole protein (10).
have found similar results with this amino-terminal region of DBP (data not shown). It may be that other sequences in these proteins inhibit the amino-terminal activation domain, which the PAR domain overcomes. Alternatively, the isolated amino-terminal activation domain may interact with a different part of the transcriptional machinery compared with the intact protein. The amino-terminal and PAR domains may represent separate contact points for the same protein or may contact two different proteins where both are required for function. The conserved nature of both the amino-terminal and PAR domains of all three known PAR domain proteins, coupled with their similar tissue distribution, suggests that they interact with the same protein or a closely related factor.

Transcriptional activation occurs either through the direct recruitment of parts of the transcriptional machinery such as TFIID or in conjunction with coactivators. The coactivator p300 is known to interact with a wide variety of bZIP factors including CREB (18), c-Jun (19), and CCAAT/enhancer binding protein β (20). It functions as a histone acetyltransferase (21) and may act to create a chromatin environment favorable for transcription (22). The p300 protein has been implicated in the regulation of differentiation in a variety of systems (23) and has been shown to undergo increased phosphorylation in response to differentiation (24). The ability of p300 to overcome E1A-mediated inhibition of DBP transactivation strongly suggests that this coactivator is required for the transcriptional activity of DBP. The inability of CBP to complement the E1A-mediated repression of DBP clearly differentiates the functions of p300 and CBP in this context. The dependence of DBP transactivation on a coactivator, rather than directly recruiting the transcriptional machinery, implies a requirement for regulation of this activity. This may be linked to changes in gene expression associated with terminal differentiation of the liver when DBP is exclusively expressed (1). The ability of DBP and HLF expression to increase DBP transactivation and to up-regulate the DBP promoter (25) suggests that some form of autoregulation of PAR protein activity involving p300 may occur in liver cells.

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