Cyclin D1 Represses the Basic Helix-Loop-Helix Transcription Factor, BETA2/NeuroD*

Expression of the hormone secretin in enteroendocrine cells is restricted to the nondividing villus compartment of the intestine, implying that terminal differentiation is linked to cell cycle arrest and that differentiation is repressed in actively proliferating cells. We have shown previously that the basic helix-loop-helix protein, BETA2/NeuroD, induces cell cycle withdrawal in addition to increasing secretin gene expression. A number of transcription factors important for differentiation are repressed by D cyclins. Repression by D cyclins appears to be independent of its effects on the cell cycle. We show that cyclin D1 represses BETA2/NeuroD-dependent transcription of the secretin gene. Examination of cyclin box mutants shows that repression is unrelated to Cdk4 activation. Although cyclin D1 and BETA2 associate in vivo, they do not directly interact. Cyclin D1 may be recruited to BETA2 by binding to the C-terminal domain of the p300 coactivator, downstream from the BETA2-binding site. In the small intestine, cyclin D1 expression occurs only in the actively proliferating crypts of Lieberkuhn but not in villi. Thus repression by cyclin D1 may serve to prevent secretin gene transcription from occurring in relatively immature epithelial progenitor cells.

The mammalian small intestinal epithelium undergoes continuous self-renewal and differentiation along the crypt-villus axis. Cellular proliferation is restricted to the crypt compartment where totipotent stem cells reside. As cells migrate up the crypt-villus axis, proliferating cells cease to divide and differentiate into each respective epithelial cell lineage. Secretin-expressing enteroendocrine cells represent a subpopulation of enteroendocrine cells found exclusively in the villus epithelium. Tritiated thymidine labeling studies showed that expression of the hormone secretin occurs only after cells cease dividing (1). These characteristics suggest that factors involved in cell cycle regulation may also regulate the terminal differentiation program of the secretin cell.

We have shown previously (2) that BETA2/NeuroD, a basic helix-loop-helix protein, is essential for expression of the secretin gene. BETA2 null mice failed to develop secretin- and cholecystokinin-expressing cells, whereas the remaining enteroendocrine cell populations appeared relatively unaffected (3). BETA2 binds to an E box sequence in the secretin gene heterodimerized with one of several ubiquitously expressed products of the E2A gene (4) and functionally associates with the p300/CREB-binding protein coactivators (5). Overexpression of BETA2 in cell lines induces cell growth arrest. Observations in BETA2 null mice further support a role for BETA2 in inducing cell cycle arrest in endocrine cells in the small intestine. Normally quiescent cells stained for S phase markers indicating reentry into the cell cycle in the absence of a functional BETA2 gene (5). Taken together, these findings suggest that BETA2 plays a key role in the coordination of terminal differentiation of secretin cells with their cell cycle withdrawal.

The stratification of proliferation and differentiation along the crypt-villus axis of the intestine suggests a close relationship between execution of a terminal differentiation program and expression of proteins that regulate G1/S progression. The early G1 cyclins D1, D2, and D3 have a well established role in progression through the G1 phase of the cell cycle. D-type cyclins assemble with cyclin-dependent kinases (Cdks)1 to phosphorylate target proteins such as pRb. Cyclins have been identified as positive regulators of the cell cycle. In the murine small intestine, cyclin D1 expression has been studied in most detail and is limited to cells within the crypt compartment, whereas its partner, Cdk4, is more widely distributed in both the crypt and nonproliferating villus compartment (6). This observation suggests that the cyclin functions as the rate-limiting partner in the assembly of active kinase in the intestine as has been suggested for other tissues (7).

In addition to their established role in regulating the activity of Cdk4/6, D-type cyclins appear to modify the activity of transcription factors, a function that does not require an association with Cdks and therefore does not involve regulation of target protein phosphorylation. These functions may coordinate gene transcription with cell cycle activity. D cyclins increase the activity of the estrogen receptor, an activity that may help stimulate proliferation of breast tissue with estrogen-induced mammary differentiation (8, 9). More commonly, D-type cyclins appear to repress transcriptional activity of transcription factors important for inducing terminal differentiation. This re...

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1 The abbreviations used are: Cdks, cyclin-dependent kinases; pRb, retinoblastoma protein; bHLH, basic helix-loop-helix; GST, glutathione S-transferase; ER, estrogen receptor; aa, amino acid; CMV, cytomegalovirus; tk, thymidine kinase; CREB, cAMP-response element-binding protein.
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In the present work, we show that transcriptional activity of BETA2 is repressed by cyclin D1. Repression does not require association of cyclin D1 with Cdks. In addition, cyclin D1 does not appear to interact directly with BETAA2 but instead associates with the C-terminal domain of the transcriptional coactivator p300, which functions as a scaffold to recruit cyclin D1 on BETAA2-containing complexes. Repression of the secretin gene by cyclin D1 in small intestinal crypts may prevent secretin gene expression in immature proliferating progenitor cells.

MATERIALS AND METHODS

Plasmids—The expression plasmids used for the transient transfection studies have been described previously: pCMV-BETA2 (5); price/CMV-cyclin D1, D2, D3 (15), and price/CMV-cyclin D1-KE (16), a gift of P. W. Hinds (Harvard Medical School, Boston); pCMV-Cdk4 (17). Plasmids encoding mutants of cDNA cyclin D1 were generated by site-directed mutagenesis from a single-stranded template prepared from price/CMV cyclin D1.

A secretin-luciferase reporter containing one copy of the secretin-enhancer spanning nucleotides -209 to +97 has been described. The luciferase reporter gene, E4tk-Luc, contains four copies of a reiterated BETAA2-binding site (-142 to -123) cloned into a SalI site upstream of the basal thymidine kinase (tk) promoter in pT81-LUC (18). The three GST-p300 plasmids containing amino acids 1-596, 744-1571, and 1572-2570 in-frame with GST moiety in the pGEX-2TK vector were provided by Y. Shi (Harvard Medical School, Boston) (19). The fragments of p300 encoding amino acids 1571-1819, 1826-2116, and 2124-2371 subcloned in the correct reading frame with GST in pGEX-3X have been described previously (5). For the Gal4-dependent reporter system, full-length p300 or amino acids 1-743, 963-1922, and 1224-1737 inserted in-frame downstream of the Gal4(1-147) DNA binding domain fragment were provided by A. Gordan (Jefferson Medical College, Philadelphia) (20).

Cell Lines and Transfections—C33A cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum and transfected by calcium phosphate precipitation (4). For transfections cells were seeded 0.1 million cells/well in 12-well multiwell plates. The next day, cells were transfected in triplicate with calcium phosphate precipitates containing 0.25 μg of luciferase reporter, 0.2 μg of beta-galactosidase expression plasmid, 0.75 μg of cyclin, 0.75 μg of Cdk4, and empty pCDNA3.1 vector to bring the total to 2.25 μg per well. For experiments using the Gal4-luciferase reporter genes, precipitates contained 1.0 μg of reporter gene, 1.0 μg of GALA-p300 fusion plasmid, 1.5 μg of cyclin expression plasmid, adding pDNA3.1 to a total of 5.0 μg per well. Cells were harvested 24 h after addition of the precipitate for luciferase assays.

Luciferase Assays—Transfected cells were harvested 24 h after addition of the calcium phosphate precipitates. Whole cell extracts were prepared from the transfected cells with 1% Triton X-100 and 100 mM potassium phosphate, pH 7.8, and 1 mM dithiothreitol. Enzymatic assays measuring luciferase activity were performed in a Monolight 2010 luminometer (Analytical Luminescence Laboratory). Results of multiple transfections were analyzed by analysis of variance.

Western Blotting—Whole cell extracts from transfected cells and cells with empty vector (see above) were precipitated with trichloroacetic acid, subjected to SDS-PAGE, and immunoblotted with rabbit antibodies (Santa Cruz Biotechnology) against cyclin D1 (SC-717, 1:200), cyclin D2 (SC-181, 1:200), and cyclin D3 (SC-182, 1:200).

Coimmunoprecipitation—IPTC cells were lysed in RIPA buffer containing 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, and 50 mM Tris-HCl, pH 8. Cell lysates were centrifuged at 14,000 × g for 10 min to pellet the debris. After percolling for 30 min with protein A/G-agarose (Roche Molecular Biochemicals), the supernatant was incubated for 1 h at 4 °C with polyclonal anti-BETA2 antibody (N-19, Santa Cruz Biotechnology), monoclonal anti-cyclin D1 antibody (HD-11, Santa Cruz Biotechnology), or normal mouse serum (1–2 μg/ml). Immunocomplexes were collected with protein A/G-agarose beads and separated by SDS-PAGE. Separated proteins were electroblotted to nitrocellulose filters and blotted with anti-cyclin D1 monoclonal antibody (HD-11, Santa Cruz Biotechnology, 1–2 μg/ml). Immunoprecipitates were assayed for Cdk4/6 activity by measuring incorporation of [3H]MP into a GST-pRB fusion protein (8).

In Vitro Transcription and Translation—Radiolabeled cyclin D1 protein was transcribed and translated in vitro from a pGem7-cyclin D1 plasmid using a TNT-reticulocyte lysate system (Promega) with Sp6 RNA polymerase and [35S]methionine (PerkinElmer Life Sciences) according to the procedures described by the supplier.

RESULTS

Cyclin D1 Is a Transcriptional Repressor of BETA2—Actively dividing cells often fail to undergo terminal differentiation until they exit the cell cycle. Repression of genes associated with differentiation by cell cycle regulatory proteins like cyclins may explain this observation. For example, cyclin D1 appears to inhibit the transcriptional activity of the myogenic basic helix-loop-helix protein, MyoD. Thus cyclin D1 may repress the expression of muscle-specific genes in actively dividing cells (12–14). Here we sought to determine whether the transcriptional activity of the bHLH protein BETA2, which is critical for expression of the secretin gene, is regulated by cyclin D1.

A secretin luciferase reporter gene containing enhancer sequences from -209 to +32 (Sec-Luc), including a single E-box-binding site for BETA2, was transiently expressed in C33A cells. Cotransfection of a BETA2 cDNA expression plasmid increased luciferase activity expressed in these cells that do not express endogenous BETA2 ~5-fold (Fig. 1A), indicating that most of the luciferase expression was BETA2-dependent. Cotransfection of a cyclin D1 expression plasmid significantly repressed reporter gene activity 2-fold in the presence of BETA2 but had no effect on the absence of BETA2 expression (Fig. 1A). As has been observed previously with other transcription factors, cyclins D2 and D3 showed reduced potency compared with cyclin D1 (Fig. 1A). Immunoblot analysis confirmed expression of 34-kDa proteins corresponding to cyclin D2 and D3 in the transfected cells, indicating that the reduced repression did not result from failure to express either protein (Fig. 1B).

One potential mechanism for the observed transcriptional repression by cyclin D1 could involve phosphorylation of intracellular target proteins by one of its associated cyclin-dependant kinases, Cdk4 or Cdk6 (21, 22). Cdk4 appeared to be a less effective inhibitor of transcription than cyclin D1 alone. Repression from overexpression of Cdk4 plus cyclin D1 was not significantly different from overexpression of cyclin D1 alone in repressing BETA2-dependent transcription of the secretin gene, raising the possibility that repression by cyclin D1 may not involve protein phosphorylation events (Fig. 1B). To clarify further whether repression by cyclin D1 occurred as a result of activation of Cdk4, we next examined whether a cyclin D1 mutant (D1-KE), with a Lys to Glu mutation at position 112 in the cyclin box, could repress BETA2-dependent transcription. This mutant D1 protein is unable to activate Cdk4, presumably due to failure to associate with the kinase (16). Immunoblot analysis showed that the D1-KE mutant was readily expressed from the transfected plasmid (Fig. 1F). The cyclin D1-KE mutant repressed transcription as effectively as native cyclin D1, suggesting that repression occurs independently of cyclin D1-stimulated Cdk4/6 enzymatic activity (Fig. 1B). As might be expected, Cdk4 had minimal effects on repression observed with the D1-KE mutant (Fig. 1B).

To confirm further that cyclin D1 was targeting NeuroD/BETA2 in the secretin gene enhancer, we constructed a reporter gene consisting of four copies of the secretin gene BETA2-binding site driving a heterologous promoter (E4tk-Luc). Cyclin D1 similarly repressed transcriptional activity of this BETA2-driver reporter gene, further indicating that transcrip-
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Fig. 1. Cyclin D1 represses BETα2-dependent secretin gene expression. C33A cells were transiently transfected with the luciferase reporter gene indicated at the top of each panel and with expression plasmids for BETα2, cyclins, and Cdk4 as indicated (A–C). Indicated cyclin D1 mutants were transiently transfected into C33A cells with a secretin-luciferase reporter gene (D). Immunoblot analysis showing expression of different D cyclins (E). Left panel shows immunoblot analysis of extracts from the transfected cells for expression of cyclins D1, D2, and D3 (lanes 1, 3, and 5) compared with untransfected cells (lanes 2, 4, and 6). Right panel shows expression of each of the cyclin D1 mutants transfection experiments of B and D. Lane 1, empty vector; lane 2, wild type (WT) cyclin D1; lane 3, cyclin D1-KE; lane 4, empty vector; lane 5, cyclin D1; lane 6, cyclin D1 LL-GCH mutant; and lane 7, cyclin D1-Δ56–161. Arrows denote the expressed proteins. Luciferase activity was measured in cell extracts 24 h after transfection. Values shown represent the mean ± S.E. of four to eight individual transfection experiments and represent the relative reporter activity normalized luciferase activity from cells transfected with the expression vector for BETα2 (*, p < 0.001 versus reporter gene + BETα2).

BETα2 and Cyclin D1 Associate Indirectly—The observed cyclin D1-mediated repression of BETα2 suggested that these proteins associate functionally in vivo. We examined cell lysates from secretin-producing and BETα2-expressing βTC3 cells for the presence of BETα2-associated cyclin D1. Immunoblotting with a cyclin D1 antibody identified an ~34-kDa band corresponding to cyclin D1 in proteins immunoprecipitated with a BETα2 antibody but not with control antisera (Fig. 2, upper part). These observations indicate that BETα2 and cyclin D1 functionally associate at native levels that occur in this pancreatic β cell line. We identified the same ~34-kDa protein that was present in immunoprecipitated proteins with cyclin D1 antibody, which served as positive control (Fig. 2, upper part).

To determine whether Cdk partners of cyclin D1 were also present in the same complexes, we assayed BETα2 immunoprecipitates for Cdk4/6 enzymatic activity. Proteins immunoprecipitated with BETα2 antibody showed no pRb kinase activity, indicating the absence of Cdk4/6 activity associated with the cyclin D1 in these complexes (Fig. 2, lower part). In control experiments, cyclin D1 immunoprecipitates yielded high levels of pRb kinase activity (Fig. 2, lower part), indicating the ability of the assay to detect the presence of active Cdk4/6. The absence of active, cyclin D1-associated Cdk4/6 in complexes immunoprecipitated by BETα2 antibody is consistent with earlier results suggesting that transcriptional repression is unrelated to Cdk4 enzymatic activity.

Although BETα2 and cyclin D1 functionally associate in vivo, the preceding commounprecipitation experiments did not indicate whether these two proteins directly interact with each other. We expressed BETα2 as a glutathione S-transferase (GST) fusion protein in bacteria and generated protein affinity resins by absorption onto glutathione-agarose beads. Full-
In vitro translated, 35S-labeled cyclin D1 was tested for its ability to bind to GST fusion proteins containing BETA2 or the N-terminal, the middle, and the carboxyl third of p300. Additional GST fusion proteins containing indicated p300 fragments were tested for their ability to capture 35S-labeled full-length cyclin D1. Input represents 10% of loaded protein.

**Fig. 3. Cyclin D1 directly binds to p300.** A, in vitro translated, [35S]-methionine-labeled cyclin D1 showed no binding to the GST-BETA2 fusion protein (Fig. 3A). Previously, we have shown (5) that the GST-BETA2 fusion protein is functional, retaining the ability to associate with E12. Thus the failure to capture cyclin D1 on the GST-BETA2 beads suggests that cyclin D1 and BETA2 do not directly interact. The presence of cyclin D1 in BETA2 immunoprecipitates suggests that cyclin D1 interacts with another BETA2-associated protein.

We have shown previously that p300 communoprecipitates with BETA2 at native levels that occur in vivo (5). Because BETA2 and cyclin D1 do not appear to interact directly, we next examined whether cyclin D1 can associate with p300, to explain its presence in BETA2 immunoprecipitates. To determine whether p300 could recruit cyclin D1 to the BETA2 containing complexes, we utilized GST-pull-down assays using three previously described GST-p300 fusion proteins containing the N-terminal, middle, and C-terminal third of p300 (Fig. 3A). Cyclin D1 bound preferentially to the fusion containing the C-terminal portion of p300 (amino acids 1572–2370), although much weaker binding was observed with the N-terminal (amino acids 1–596) or the central part of p300 (amino acids 744–1571), suggesting that cyclin D1 associates preferentially with the C-terminal third of p300.

To localize further the cyclin D1 binding domain within the C-terminal third of p300, we synthesized and tested three additional GST-p300 fusion proteins containing amino acids 1571–1819, 1826–2116, and 2124–2371 of p300 for their ability to bind to labeled cyclin D1 (Fig. 3B). The fusion protein containing amino acids 1826–2116 of p300 showed the strongest binding to cyclin D1, whereas amino acids 1571–1819 of p300 captured the labeled cyclin D1 with a much lower affinity, and p300 amino acids 2124–2371 showed no binding to cyclin D1 (Fig. 3B).

**p300 Mediates Cyclin D1 Repression on BETA2-dependent Transcription of the Secretin Gene.**—The p300 protein functionally cooperates with sequence-specific transcription factors to increase transcriptional activation in part by its direct interaction with the basal transcription machinery as well as by other mechanisms including protein acetylation. Thus p300 exhibits intrinsic transcriptional activation function when fused to the DNA binding domain of either E2 or Gal4 (20, 23). To ascertain whether p300 was the target of transcriptional repression of BETA2 by cyclin D1, we examined whether activation of a reporter gene containing multiple GAL4-binding sites by a GAL4-p300 fusion protein was repressed by cyclin D1. The GAL4-p300 fusion protein produced an ~6-fold increase in luciferase activity compared with the reporter gene alone or the GAL4-DBD alone that had no effect (Fig. 4A). Cyclin D1 reduced expression of the GAL4-luciferase reporter driven by GAL4-p300 by ~50%, much like the secretin promoter (Fig. 4A). Cotransfection of the cyclin D1-KE mutant inhibited reporter gene activity comparably to cyclin D1, suggesting that repression of p300-mediated transcription, like that of BETA2, is not related to activation of a Cdk partner.

We examined several p300 fragments fused to the GAL4 DNA binding domain for their sensitivity to cyclin D1 to map further the domains sensitive to repression by cyclin D1 (Fig. 4B). Earlier work mapped two transcriptional activation domains in p300, one at the N terminus and the other at the C-terminal third of the protein (20). The transcriptional activities of Gal4 fusion proteins containing either the N-terminal activation domain (aa 1–743) or the middle domain of p300 (aa 963–1922) were not repressed by cyclin D1. However, transcriptional activity of a fusion protein containing an internal deletion from 242–1737, containing the C-terminal 677 residues of p300, was repressed by cyclin D1, suggesting that cyclin D1 may target the C-terminal activation domain. Similarly, a fusion protein including p300 amino acids 1737–2414 was also repressed by cyclin D1 (not shown). However, as described earlier, failure to include aa 1–247 resulted in a fusion protein with greatly reduced transcriptional activity (20). These results suggest that the C terminus of p300, which has the highest cyclin D1 binding, is also the target for transcriptional repression. In contrast, the N terminus of p300, which weakly bound to cyclin D1, was not sensitive to the cyclin. Likewise, p300 (aa 963–1922), a domain with intrinsic histone acetyltransferase activity, neither bound to nor was repressed by cyclin D1.

**DISCUSSION**

D cyclins have implicated as negative regulators of transcription for a number of proteins including the myogenic basic helix-loop-helix proteins, myogenin, and MyoD (14); v-Myb (10) (but not c-Myb); the Myb-like protein, DMP1 (11); and Sp1 (24). In the case of the estrogen receptor, D cyclins appear to activate transcription especially in the absence of ligand (8, 25).

The present study shows that cyclin D1 functions as a transcriptional repressor of the basic helix-loop-helix protein, BETA2/NeuroD. The work described here adds to an increasing body of data indicating that D cyclins regulate transcription independently of their effects on the enzymatic activity of cyclin-dependent kinases. Transcriptional repression of BETA2 by cyclin D1 is notable in that unlike most other transcription factors, cyclin D1 does not directly interact with BETA2 but
instead is recruited to BETA2-containing complexes through direct binding to p300.

Initial observations showing that cyclin D1 repressed MyoD activity suggested that phosphorylation of MyoD or other proteins by activated Cdk4 might represent the mechanism of repression. Indeed, earlier observations (13) showed that overexpression of p21 overcame repression of MyoD by cyclin D1 and were consistent with the hypothesized phosphorylation as was the identification of phosphorylated forms of MyoD. Like MyoD, myogenin is inhibited by cyclin D1. However, overexpression of cyclin D1 failed to alter significantly the phosphorylation of either MyoD or myogenin (14). Furthermore, a mutant, fully active myogenin with alanine residues replacing the two phosphorylated serine residues, was inhibited comparably to the wild type myogenin (14). Thus it appears unlikely that transcriptional repression of myogenic bHLH proteins by cyclin D1 results from their phosphorylation by cyclin D1-dependent kinases (7).

In the case of other transcription factors, including BETA2 described here, v-Myb, the estrogen receptor, and DMP1, cyclin D1 mutants that cannot interact with Cdk4/6 retain their ability to regulate transcriptional activity. In all cases where examined, Cdk4 does not reproduce or enhance the effects of D cyclins, further indicating that the transcriptional changes induced by D cyclins represent an activity distinct from the regulation of Cdk4.

Generally, overexpression of Cdk4 does not reproduce the effects of cyclin D1. In the case of the ER, cotransfection of Cdk4 with cyclin D1 reversed the effect of D1 suggesting that only “free” D1 not associated with Cdk4 functioned as a transcriptional modifier (9). Likewise, Cdk4 reduces the level of association between TAF(II)250 and cyclin D1 required for Sp1 repression as does an excess of transfected pRb (24). It is not known whether activation of Cdk4 is involved in the effects of cyclin D1 on Sp1 although reversal of repression by Cdk4 argues against this possibility. The failure to identify any Cdk 4/6 activity in BETA2 immunoprecipitates containing cyclin D1 may indicate that only free D1 is available for transcriptional regulation. However, the failure to overcome repression with Cdk4 suggests that BETA2/p300 and Cdk4 do not directly compete for limiting amounts of cyclin D1.

Of the three D-type cyclins, D1 appears to be the strongest repressor of BETA2. The observed potency of D1 has been noted in several other cases as well. In one case, the activation of the estrogen receptor was highly specific for cyclin D1 (9), whereas in a second report, D1 was the most potent activator of the ER with less activation seen with D2 and D3 (8). Cyclins D1 and D2 were capable of inhibiting MyoD, whereas D3 showed little effect (13). In the case of v-Myb, all three D cyclins were inhibitory with D1 > D2 > D3. All three D cyclins functioned as equipotent inhibitors of DMP1 (11).

The relative potency of cyclin D1 versus D2 and D3 as transcriptional modifiers was not anticipated given the homology of D2 and D3 with D1 (71 and 63% similar, respectively). Of note is the central region, including the cyclin box, is highly conserved among the three. However, this region appears to be dispensable for transcriptional repression. With the exception of the LXCXE motif at the N terminus, the N- and C-terminal domains of cyclins D2 and D3 are much less highly conserved with D1. The N terminus of cyclin D1 (amino acids 1–100)
appears to be sufficient for its association with TAF(II)110 in order to repress Sp1 (24). The region of cyclin D1 required for repression has been examined for MyoD as well. Similar to the present work, mutations in the N-terminal pRb binding consensus sequence in cyclin D1 did not affect transcriptional repression. However, a C-terminal truncation mutant containing only the first 202 amino acids of cyclin D1 did not repress MyoD (12).

There is no single mechanism to account for how cyclins regulate transcription, although most evidence indicates that activation of cyclin-dependent kinases is not critical. In the present work, we found that transcriptional repression of BETA2 by cyclin D1 is indirect, unlike most other transcription factors studied that directly interact with cyclin D1. Instead, cyclin D1 appears to target p300 by associating with the C-terminal third of the protein thus representing a new pathway for transcriptional regulation by cyclin D1. Although we showed weak binding of cyclin D1 in vitro to other regions of p300, it does not appear that these potential interactions contribute to repression. Thus cyclin D1 does not appear to target an intrinsic activation domain in the N terminus of p300 nor the central region that contains histone acetyltransferase (HAT) activity.

The intrinsic transcriptional activating activity in the C-terminal third of p300 appears to be repressed by cyclin D1. This region of p300 contains the binding sites for pCAF, E1A, and BETA2. However, the strongest cyclin D1 binding activity was localized about 200 residues further toward the C terminus of p300, making it unlikely that there is direct competition with BETA2 or pCAF. Thus p300 appears to function as a scaffold to recruit cyclin D1 to BETA2-containing complexes. Repression of RelA activity by cyclin E similarly does not involve a direct interaction. Cyclin E binds to the C terminus of p300, a site that is distinct from the RelA-binding site at the N terminus of the coactivator (26). The effects of cyclin D1 on Sp1 also appear to be indirect, mediated through association with TAF(II)110. Cyclin D1 may displace pRb from TAF(II)110, thereby disrupting interaction with Sp1-TAF(II)110 with TAF(II)110 (24).

Other transcription factors appear to be regulated differently by D cyclins. Direct association of D cyclins with DMP1 appears to inhibit transcription by disrupting the formation of DMP1-DNA complexes (11). However, we observed no reduction in the ability to form DNA complexes in electrophoretic mobility shift assays in the presence of added cyclin D1. Similarly, cyclin D1 had no effect on the ability of v-Myb, MyoD, myogenin, and the estrogen receptor to bind to DNA.

It has been suggested that the mechanism for cyclin D1 repression of MyoD involves cyclin D1-mediated nuclear translocation of Cdk4, allowing Cdk4 to bind to a 15-amino acid motif in the C terminus of MyoD (7, 27). Cdk4-associated MyoD appears to be unable to bind to DNA with its dimerization partner E1C. In contrast, myogenin and BET2A both lack the Cdk4-binding site and are not repressed by Cdk4. The failure to increase repression in cells cotransfected with both Cdk4 and cyclin D1 as well as the potency of the cyclin D1-KE mutant also argue against a similar mechanism for BET2A. Thus cyclin D1-directed nuclear translocation of Cdk4 might represent a specific mechanism for inhibition of MyoD not applicable to other bHLH proteins.

Activation of the estrogen receptor by cyclin D1 appears to occur through a mechanism that is distinct from transcriptional inhibition. Cyclin D appears to function as a bridging molecule to assemble ternary complexes with the ER. In one case, pCAF may be recruited to the ER by cyclin D1 to potentiate the effects of cyclin D1 (28). In addition, leucine-rich motifs in the C-terminal third of cyclin D1 directly bind to the steroid hormone coactivator, SRC1. Ternary complexes of ER, D1, and SRC1 allow the ER to bind to DNA in the absence of ligand and to activate transcription (25). It would appear unlikely that recruitment of SRC1 is involved in repression of BETA2 as SRC1 has no effect on BET2A-dependent transcription. It is notable that the region of p300 we identified that binds to cyclin D1 may overlap the region known to associate with SRC1.

Cyclin D1 repression of BET2A may contribute to the temporal and spatial differentiation of secretin-expressing enteroendocrine cells in the intestine. Each of the four epithelial cell lineages in the intestine, including enteroendocrine cells, arises from a common totipotent stem cell in the intestinal crypts. Cells migrate in vertical sheets up the crypt-villus axis into the villus compartment where they stop dividing and terminally differentiate. Cyclin D1 expression is restricted to proliferating cells in the intestinal crypts and disappears in the villi. Secretin-expressing enteroendocrine cells are found only in the villi and never stain for markers of S phase. Thus, expression of cyclin D1 in the intestinal crypts may play a role in delaying expression of the secretin gene in immature progenitor cells until they further differentiate and enter the villus compartment. The importance of transcriptional repression by cyclin D1 in preventing premature differentiation of other epithelial lineages in the proliferating crypt compartment has not been studied. A number of transcription factors implicated in enterocyte differentiation, including GATA-4, -5, -6, and GKLF, are not appreciably expressed in the lower crypt and are unlikely to be repressed by cyclin D1 (29, 30). However, HNF-1α, Cdx-1, and Cdx-2 are expressed in the lower crypt and are candidates for repression by cyclin D1 (31, 32). Such a role for cyclin D1 remains entirely speculative at this time.

Acknowledgments—We thank Phil Hinds (Harvard Medical School, Boston), Yang Shi (Harvard Medical School, Boston), and Antonio Giordano (Jefferson Medical School, Philadelphia) for generously providing plasmids as noted under “Materials and Methods.”

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