A Role for Cell Cycle-Regulated Phosphorylation in Groucho-Mediated Transcriptional Repression

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‡ Supported by a CIHR Postdoctoral Fellowship and a J.T. Costello Postdoctoral Fellowship from the Montreal Neurological Institute

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SUMMARY

Transcriptional corepressors of the Groucho/transducin-like Enhancer of split (Gro/TLE) family are involved in a variety of cell differentiation mechanisms in both invertebrates and vertebrates. They become recruited to specific promoter regions by forming complexes with a number of different DNA-binding proteins thereby contributing to the regulation of multiple genes. To understand how the functions of Gro/TLE proteins are regulated, it was asked whether their ability to mediate transcriptional repression might be controlled by cell cycle-dependent phosphorylation events. It is shown here that activation of p34<sup>cdc2</sup> kinase (cdc2) activity with okadaic acid is correlated with hyperphosphorylation of Gro/TLEs. Moreover, pharmacological inhibition of cdc2 activity results in Gro/TLE dephosphorylation. In agreement with these findings, a purified cdc2-cyclin B complex can directly phosphorylate Gro/TLEs <em>in vitro</em>. Two separate Gro/TLE domains, the CcN and SP regions, contain sequences that are phosphorylated by cdc2. Deletion of these sequences is correlated with loss of Gro/TLE phosphorylation by cdc2 <em>in vitro</em> and okadaic acid-induced Gro/TLE hyperphosphorylation <em>in vivo</em>. In addition, Gro/TLEs are phosphorylated during the G2/M phase of the cell cycle and this is correlated with a decreased nuclear interaction. Finally, the transcription repression ability of Gro/TLEs is enhanced by pharmacological inhibition of cdc2. Taken together, these results demonstrate that Gro/TLE proteins are phosphorylated as a function of the cell cycle and implicate phosphorylation events occurring during mitosis in the negative regulation of Gro/TLE activity.
INTRODUCTION

In both invertebrates and vertebrates, transcriptional corepressor proteins of the Groucho/transducin-like Enhancer of split (Gro/TLE) family play crucial roles in the regulation of a variety of cell differentiation mechanisms. In particular, Drosophila Gro is required for sex determination, segmentation, and neural development (1). Vertebrate Gro/TLE proteins contribute to the regulation of neuronal development (2), patterning of the neural tube (3, 4), skeletogenesis (5, 6), hematopoiesis (7-9), and myogenesis (10, 11).

Gro/TLE proteins have no intrinsic DNA-binding activity but can be targeted to specific gene regulatory regions due to their ability to interact with a number of different DNA-binding transcription factors. Examples of these include bHLH proteins of the Hes family (12-17), Runt-homology domain proteins of the Runt/RUNX family (7, 18, 19), homeodomain factors containing engrailed homology region 1 motifs (3, 20-24), winged-helix domain transcription factors (23, 25), and HMG-box proteins (7, 9). By virtue of these multiple interactions, the general transcription repression activity of Gro/TLE proteins can be recruited in context-dependent manners to specific target genes.

Transcriptional repression by Gro/TLE family members is thought to be mediated by at least two mechanisms. Oligomeric Gro/TLEs can interact with both histones (26, 27) and histone deacetylases (23, 28-30), consistent with a process in which recruitment of Gro/TLEs to DNA may result in the removal of acetyl groups from the amino-terminal domains of histones. In turn, this is thought to result in the establishment of a compact chromatin structure that is not amenable to gene activation. In addition, recent work has raised the possibility that Gro/TLE proteins may target the activity of the basal transcriptional machinery through interaction with
the TFIIE factor (31). This possibility is consistent with the observation that the protein TUP1, a general corepressor thought to represent the functional analog of Gro/TLEs in yeast (27, 32), interacts with RNA polymerase II holoenzyme components and can repress transcription in vitro (33, 34).

Little is presently known about the mechanisms that control the transcription repression ability of Gro/TLEs. In that regard, recent studies have implicated phosphorylation events induced by cofactor binding in promoting Gro/TLE activity (17). In particular, interaction of Gro/TLEs with the Hes and RUNX family members, Hes1 and RUNX1, results not only in the recruitment of Gro/TLEs to DNA but also in their hyperphosphorylation. Hes1-induced hyperphosphorylation is correlated with an increase in the affinity of Gro/TLEs with the nuclear compartment, likely the result of the establishment of a strong interaction with chromatin components. Protein kinase CK2 is involved in the Hes1-induced hyperphosphorylation of Gro/TLEs, and inhibition of protein kinase CK2 activity reduces the transcription repression ability of Gro/TLEs (17). These findings underscore the importance of phosphorylation events in the functions of Gro/TLE proteins.

To examine further the mechanisms involved in the regulation of Gro/TLE activity, we have tested the possibility that these factors may be dynamically phosphorylated during the cell cycle. Our results show that Gro/TLEs are phosphorylated by p34\(^\text{cdc2}\) kinase (cdc2), a master regulator of the G\(_2\)/M transition and entry into mitosis (35). This is in agreement with the presence of conserved motifs resembling phosphorylation sites for cdc2 within all Gro/TLE family members. Our findings also suggest that mitotic phosphorylation of Gro/TLEs reduces their ability to mediate transcriptional repression by weakening their interaction with nuclei.
Together, these observations provide new insights into the regulation of the phosphorylation state and functions of Gro/TLE proteins.
EXPERIMENTAL PROCEDURES

Cell Culture and Reagents - Drosophila S2, rat ROS17/2.8 and human HEK 293 (‘293A’), HeLa and Jurkat cells were cultured as described previously (16, 19, 36). Purified cdc2-cyclin B complex was purchased from New England Biolabs. Okadaic acid, roscovitine and olomoucine (Calbiochem) were dissolved in dimethyl sulfoxide prior to use. Nocodazole was obtained from Sigma. Purified histone H1 was from Roche. Antibodies used in this study were obtained as follows: anti-(pTyr15)cdc2 (New England Biolabs), anti-Drosophila Gro (36, 37), panTLE (2, 16, 17, 38), anti-Gro/TLE1 (36, 39), anti-histone H3 (16, 17), anti-HDAC2 (Zymed Labs. Inc.), and anti-cdc2, -GAL4bd, and -GST (Santa Cruz Biotechnology).

Okadaic Acid Treatment and Pharmacological Inhibition of cdc2- ROS17/2.8 or 293 cells were grown in 6-well plates in the presence or absence of okadaic acid (43), roscovitine (44), or olomoucine (45) as described previously and at the concentrations indicated in the figure legends. Cells were then collected, and whole-cell lysates were prepared and subjected to western blotting analysis of either endogenous Gro/TLE or transfected GAL4bd-Gro/TLE1 proteins. Expression of GAL4bd-Gro/TLE1(full-length) was driven by the previously described (15, 17) plasmid pcDNA3-GAL4bd-Gro/TLE1. For analysis of Gro/TLE1 deletion mutants, the plasmids pcDNA3-GAL4bd-Gro/TLE1(Δ258-268), GAL4bd-Gro/TLE1(Δ285-335), and GAL4bd-Gro/TLE1(Δ258-335) were obtained by first generating the sequences containing the appropriate deletions using PCR-based strategies (information on oligonucleotide primers is available upon request), followed by subcloning into pBluescript(SK) plasmid and DNA sequencing. The verified deletion products were then subcloned into pcDNA3-GAL4bd-Gro/TLE1 digested with BstEII and SacII, replacing the corresponding wild type sequence.
Metabolic Labeling and Cell Synchronization - HeLa cells were labeled with $[^{32}\text{P}]\text{Pi}$ as described previously (36). For cell synchronization at G2/M, exponentially growing HeLa cells were treated for 10-16 hr with nocodazole as described (40-42). After this time, mitotically enriched cells that were loosely attached or floating were collected by gentle pipetting while adherent cells were not collected. This protocol was shown to yield greater than 80% of cells with a DNA content corresponding to the G2/M phase, with most of these cells being in mitosis (42). Cultures enriched for cells arrested at the G1/S transition were obtained by treatment with 10 mM hydroxyurea (41), followed by removal of floating or loosely attached cells and recovery of the strongly adherent cells.

In Vitro Phosphorylation of Immunoprecipitated or Bacterially Purified Gro/TLE Proteins – For in vitro phosphorylation assays with purified proteins, the following plasmids were used for the bacterial expression and purification of fusion proteins of GST and individual Gro/TLE domains (see Ref. 38 for a description of these domains). Constructs pGEX2-Gro/TLE1(Q) (15, 32), pGEX1-Gro/TLE1(SP) (19), and pGEX3-Gro/TLE3(WDR) (38) have been described previously. Constructs pGEX2-Gro/TLE1(GP), pGEX2-Gro/TLE1(CcN), pGEX1-Gro/TLE1(SP-N) (encoding the amino-terminal half of the SP domain, i.e., amino acids 290-374), pGEX2-Gro/TLE1(CcN/SP-N) (encoding the CcN domain and the N-terminal half of the SP domain; i.e., amino acids 199-374), and pGEX2-Gro/TLE1(CcN/SP-NA258-335) were generated by PCR amplification of the regions of interest as described previously (15, 32), followed by subcloning into the indicated pGEX vectors. For phosphorylation assays with native Gro/TLE proteins, Drosophila Gro or human Gro/TLE1 were immunoprecipitated from S2 or HeLa whole-cell lysates, respectively, as described previously and in the presence of 1% Triton
X-100 (17, 36). Immunoprecipitates were washed with ice-cold buffer D [50 mM HEPES (pH 7.6), 200 mM NaCl, 1% Triton X-100]. Each kinase assay contained either roughly 50 ng of purified fusion protein or the product of one immunoprecipitation. Samples were resuspended in buffer E [50 mM Tris-HCl (pH 7.6), 10 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1% Triton X-100, 200 μM ATP] containing 200 μCi/ml of [γ³²P]-ATP in the presence of 2 units of purified cdc2-cyclin B complex per reaction. After incubating for 30 min at 37 °C, reactions were terminated by the addition of 2x SDS-PAGE sample buffer and incubation at 65 °C for 5 min, followed by gel electrophoresis and either autoradiography or Western blotting analysis. Alternatively, the products of kinase reactions with bacterially purified fusion proteins were loaded onto SpinZyme phosphocellulose units (Pierce) and centrifuged at 3000 x g for 30 sec. The filters were then extensively washed with 75 mM phosphoric acid and dried. Bound radioactivity was measured in a scintillation counter.

Subcellular Fractionation – Preparation of whole-cell lysates was as described previously (17, 26, 36). Post-nuclear supernatant and nuclear fractions were prepared by first washing the cells with ice-cold phosphate-buffered saline, followed by resuspension for 10 sec in ice-cold buffer A [20 mM HEPES (pH 7.6), 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM dithiotreitol, and 1x “Complete” protease inhibitor cocktail (Roche)]. Cells were quickly collected by centrifugation, resuspended in 10 packed cell pellet volumes of buffer A and incubated on ice for 10 min, followed by trituration through a 25-gg needle. The homogenate received 0.5 cell pellet volumes of buffer B [50 mM HEPES (pH 7.6), 1 M KCl, 0.5 mM EDTA, and 1x “Complete” protease inhibitor cocktail] and was mixed thoroughly, followed by centrifugation at 300 g for 1 min to remove debris. The supernatant was collected and centrifuged at 1,500 g for 15 min to yield a supernatant fraction (post-nuclear supernatant) and a crude nuclear pellet. The nuclear pellet was
washed twice by resuspending in buffer A and centrifuging at 1,500 g for 15 min. This was followed by resuspension in 3-5 volumes of buffer C [20 mM HEPES (pH 7.6), 500 mM NaCl, 0.5 mM EDTA, 1% Triton X-100, and 1x “Complete” protease inhibitor cocktail]. After centrifugation at 1,500 g for 15 min, the supernatant fraction was collected (nuclear extract). Whole-cell lysates, post-nuclear supernatants, and nuclear extracts were subjected to SDS-PAGE, followed by either autoradiography or Western blotting as described (16, 17, 23, 36).

Transcription Assays - HeLa or 293A cells were transfected using the Superfect reagent (Qiagen) as described (16, 17). The total amount of transfected DNA was adjusted in each case at 2 µg per well using pcDNA3. Transcription assays were performed using 0.5 µg of reporter construct p5xGAL4UAS-tk-luciferase in the presence or absence of plasmids pcDNA3-GAL4bd, pcDNA3-GAL4bd-Gro/TLE1, or pGAL4bd-HDAC4 (46) (0.1 µg). In each case, 0.5 µg of the β-galactosidase reporter plasmid, pRSV-βgal, was used to normalize for transfection efficiency. Twenty-four hours after transfection cells were treated or not treated with roscovitine (10 µM) or olomoucine (20 µM), cultured for a further 24 hr, and then subjected to determination of luciferase activity as described (15, 16, 23). Results were expressed as mean values ± the standard deviation.
RESULTS

Okadaic Acid-Induced Phosphorylation of Gro/TLE Proteins Concomitant with Activation of cdc2 – Gro/TLE family members can translocate on their own to the nucleus where they contribute a transcription corepression activity to a number of different DNA-binding proteins (1-9). Previous immunofluorescence studies have shown that Gro/TLEs are localized to nuclei during interphase and interact with nuclear compartments like chromatin and the nuclear matrix (6, 17, 26, 38). Since the functions of several transcription factors are negatively regulated during mitosis by phosphorylation mechanisms that control their interaction with chromatin and/or other nuclear structures, we examined whether Gro/TLEs are phosphorylated at mitosis. This possibility was also suggested by the presence of several possible phosphorylation sites for cdc2 within all Gro/TLE family members (38). Endogenous Gro/TLE expression and phosphorylation was determined in asynchronously growing rat ROS17/2.8 osteosarcoma cells cultured in the absence or presence of increasing doses of the cell-permeable compound okadaic acid. This compound was shown to selectively inhibit protein phosphatase 2A and indirectly activate cdc2 by inducing phosphorylation of cdc25 (47-49). The chosen concentrations of okadaic acid have been shown previously to selectively activate cdc2 and not cell cycle-dependent kinases active at G1/S like cdk2, cdk4, or cdk6 (47, 49). Moreover, okadaic acid did not activate a large number of other kinases when tested at the concentrations used in our studies (43, 50). We found that increasing amounts of okadaic acid caused a progressive retardation of the Gro/TLE electrophoretic mobility, resulting in the appearance of more slowly migrating forms (Fig. 1A, cf. lanes 1 and 5-7). These forms corresponded to hyperphosphorylated Gro/TLE species because they were not observed after treatment with calf intestinal phosphatase (Fig. 1D,
cf. lanes 3 and 4). No similar changes were observed when cells were treated with dimethyl sulfoxide (carrier) alone (Fig. 1A, lanes 2-4). We next determined whether the okadaic acid-induced phosphorylation of Gro/TLEs was correlated with cdc2 activation by using a phospho-specific monoclonal antibody that recognizes only inactive cdc2 (phosphorylated at Tyr15) (51). Although the overall level of cdc2 (detected with a nonphospho-specific antibody) was unchanged by treatment with okadaic acid (Fig. 1B, cf. lanes 1 and 5-7), the amount of inactive cdc2 decreased in the presence of okadaic acid in a dose-dependent manner, indicative of cdc2 activation (Fig. 1C, cf. lanes 1 and 5-7). In contrast, dimethyl sulfoxide alone had no effects (Fig. 1C, lanes 2-4). Taken together, these results show that okadaic acid induces the hyperphosphorylation of Gro/TLE proteins concomitant with the activation of cdc2. This suggests that cdc2 is involved in Gro/TLE phosphorylation.

Phosphorylation of Gro/TLE Proteins by cdc2 In Vivo and In Vitro – To examine if cdc2 can phosphorylate Gro/TLEs, the ability of okadaic acid to induce Gro/TLE phosphorylation was examined in cells cultured in the absence or presence of two cell permeable pharmacological inhibitors of cdc2. Both olomoucine (45) and roscovitine (44) reduced the okadaic acid-induced phosphorylation of Gro/TLEs in a dose dependent manner, resulting in faster electrophoretic mobility (Figs. 2A and B, cf. lanes 3-6). The chosen concentrations of these pharmacological inhibitors were shown to maintain viability of the cells and not induce irreversible arrest in G2 phase (42, 44). Importantly, both of these inhibitors also increased Gro/TLE mobility in the absence of okadaic acid (Figs. 2A and B, cf. lanes 1 and 2). Together, these findings suggest further that cdc2 is involved in Gro/TLE phosphorylation.

We next tested if a purified cdc2-cyclin B complex could phosphorylate Gro/TLE proteins by performing in vitro kinase assays. Gro/TLE proteins were immunoprecipitated from
either Drosophila S2 or mammalian HeLa cells. In both cases, incubation with cdc2-cyclin B resulted in the phosphorylation of immunoprecipitated Gro/TLE proteins (Figs. 3A, lane 2, and 3C, lane 1, see long arrow). No phosphorylation products were observed after immunoprecipitation with control antibodies (Figs. 3A, lane 3, and 3C, lane 2), or when purified cdc2-cyclin B was omitted (Fig. 3A, lane 1). Phosphorylation of cyclin B, which is known to be an intra-complex substrate of cdc2 (51), was also detected (Figs. 3A and C, see open arrow). Taken together, these results implicate cdc2 in the phosphorylation of Gro/TLE proteins.

Mapping of Gro/TLE Domains Phosphorylated by cdc2 – To both demonstrate further that purified cdc2 can directly phosphorylate Gro/TLE proteins and to identify the domains of the latter that are targeted by this kinase, individual Gro/TLE domains (38 and Fig. 4A) were isolated from bacteria as fusion proteins with GST (Fig. 4B). In vitro phosphorylation assays showed that the CcN domain was preferentially phosphorylated by purified cdc2-cyclin B (Fig. 4C, lane 7); a weaker but clearly detectable phosphorylation of the SP domain was also observed (Fig. 4C, lane 9). GST alone and fusion proteins of GST and other Gro/TLE domains were not phosphorylated by cdc2 even though they were properly expressed (Figs. 4B and C). Our mapping studies showed further that cdc2 phosphorylated sequences located within the amino-terminal half of the SP domain of Gro/TLE1 (amino acids 290-374) (Figs. 5A and B, lane 5). We next generated a fusion protein containing both the CcN and SP-N regions. This protein was phosphorylated in vitro by cdc2 (Figs. 5C and D, lane 3); phosphorylation incorporated 0.20±0.08 pmol of phosphate/pmol of fusion protein (n=3). More importantly, deletion of amino acids 258-335, which contain putative cdc2 phosphorylation sequences (see Fig. 6A below) significantly reduced phosphorylation by cdc2 (0.017±0.015 pmol of phosphate/pmol of fusion protein),
protein; n=3) (Figs. 5C and D, lane 5), indicating that residues 258-335 are targets for cdc2 activity. Thus, these results show that cdc2 can directly phosphorylate Gro/TLE proteins in vitro.

To determine if the sites phosphorylated by cdc2 in vitro correspond to sites that are phosphorylated in vivo in response to okadaic acid treatment, a panel of Gro/TLE1 deletion mutants were generated in which regions containing potential cdc2 phosphorylation sites were removed (Fig. 6A). These proteins were expressed in transfected cells as fusion proteins with GAL4bd and the effect of okadaic acid on their phosphorylation examined as described in Figures 1 and 2. Similar to endogenous Gro/TLEs, full-length Gro/TLE1 exhibited an increase in phosphorylation in the presence of okadaic acid, and this hyperphosphorylation was antagonized by olomoucine, suggesting that it involved cdc2 activity (Fig. 6B, cf. lanes 4-6). In contrast, Gro/TLE1(Δ258-268), lacking the putative cdc2 target sequence SSPRASPAHSPR in the CcN domain, exhibited only a small gel retardation in the presence of okadaic acid. Importantly, olomoucine treatment did not result in a detectable shift to a faster mobility, suggesting the loss/reduction of phosphorylation events mediated by cdc2 as a result of the deletion under study (Fig. 6B, cf. lanes 1-3). These observations suggest that the deleted region is phosphorylated in vivo by cdc2, consistent with the results of in vitro phosphorylation assays shown in Figures 4 and 5. Similar studies showed that deletion of amino acids 285-335 only marginally reduced the okadaic acid-induced gel retardation of Gro/TLE1 (Fig. 6C, lanes 3 and 4), whereas no significant electrophoretic shift was observed when GAL4bd-Gro/TLE1(Δ258-335), which combined both of the previous deletions, was examined (Fig. 6C, lanes 5 and 6). Taken together, these findings show that cdc2 phosphorylates Gro/TLE1 in vitro at sites within the CcN domain that are also phosphorylated in vivo following okadaic acid treatment, suggesting that cdc2 is involved in Gro/TLE phosphorylation in vivo.
**Phosphorylation of Gro/TLE Proteins During the Cell Cycle** – To determine whether Gro/TLEs are differentially phosphorylated at various stages of the cell cycle, HeLa cells were arrested at either the G1/S or G2/M transition using hydroxyurea or nocodazole, respectively, followed by metabolic labeling with $[^{32}\text{P}]\text{Pi}$, preparation of whole-cell extracts and immunoprecipitation with anti-Gro/TLE1 antibodies. A roughly 90-kDa phosphorylated form of Gro/TLE1 was present in both G1/S- and G2/M-arrested cells (Fig. 7, lanes 2 and 4, see short arrow). In contrast, a more slowly migrating phosphorylated form of roughly 95 kDa was observed only in G2/M cells (Fig. 7, lane 4, see long arrow). This change in Gro/TLE phosphorylation was correlated with the activation of cdc2 in G2/M cells (see Figures 8B and C below). These results show that Gro/TLE1 is differentially phosphorylated during the cell cycle and becomes hyperphosphorylated at the G2/M transition.

**Cell Cycle-Dependent Phosphorylation of Gro/TLE Proteins is Correlated with Changes in Nuclear Association** – To determine if cell cycle-dependent phosphorylation events might be involved in regulating the nuclear association of Gro/TLEs, HeLa cells were arrested at the G1/S or G2/M transition, followed by isolation of nuclear and post-nuclear supernatant fractions and analysis of Gro/TLE localization to these fractions by Western blotting. In G1/S cell-enriched cultures, most Gro/TLE proteins were found in the nuclear fraction, indicative of a strong association with nuclei (Fig. 8A, cf. lanes 1 and 2). A similar preferential association with nuclei was observed when the nuclear proteins HDAC2 (Fig. 8D, lane 2) and histone H3 (Fig. 8E, lane 2) were tested. In G2/M cells, a considerable amount of Gro/TLEs appeared to be weakly bound
to, or possibly excluded from, nuclei and was recovered in the non-nuclear fraction (Fig. 8A, cf. lanes 3 and 4). Importantly, we observed that the hyperphosphorylated Gro/TLE forms present only in G2/M-arrested cells were predominantly localized to the non-nuclear fraction (Fig. 8A, lane 3, see arrow). In contrast, HDAC2 (Fig. 8D, lane 4) and histone H3 (Fig. 8E, lane 4) did not behave like Gro/TLEs and were found in the nuclear fraction from G2/M-arrested cells.

Examination of the expression of cdc2 phosphorylated at Tyr 15 (inactive cdc2) showed that this kinase was inactive and predominantly localized to the non-nuclear fraction in G1/S cells (Fig. 8B, lane 1). In contrast, inactive cdc2 was not observed in G2/M cells (Fig. 8B, lanes 3 and 4). Reprobing with phosphorylation-state independent antibodies revealed that this decrease in inactive cdc2 was not the result of cdc2 degradation because the protein was expressed in G2/M cells, where it associated preferentially with the nuclear compartment (Fig. 8C, lane 4). Taken together with the results depicted in Figure 7, these findings show that Gro/TLEs are differentially phosphorylated during the cell cycle and that the hyperphosphorylated Gro/TLE proteins present in G2/M cells interact weakly with, or are excluded from, the nuclear compartment. These changes in Gro/TLE phosphorylation and nuclear interaction are correlated with the activation of cdc2 in G2/M cells, suggesting that this kinase is involved in Gro/TLE phosphorylation at mitosis.

Promotion of the Transcription Repression Activity of Gro/TLE1 by Pharmacological Inhibition of cdc2 Activity – The correlation between increased phosphorylation at the G2/M transition and decreased nuclear association of Gro/TLEs suggested that phosphorylation events involving cdc2 may play a role in the negative regulation of Gro/TLE-mediated transcriptional
repression. We therefore tested whether repression by Gro/TLE proteins might be enhanced by the pharmacological inhibition of cdc2. Since Gro/TLEs have no intrinsic DNA-binding ability, we examined the GAL4bd-Gro/TLE1 fusion protein. This fusion protein was found preferentially associated with the nuclear fraction in G1/S cells (Fig. 9A, lane 2) but was mostly localized to the non-nuclear fraction in G2/M cells (Fig. 9A, lane 3). This suggests that the nuclear association of this fusion protein changes as a function of the cell cycle in a manner analogous to the behavior of endogenous Gro/TLEs. More importantly, pharmacological inhibitors of cdc2 increased the electrophoretic mobility of GAL4bd-Gro/TLE1 present in the non-nuclear fraction from G2/M cells, suggesting that this fusion protein is phosphorylated by cdc2 (Fig. 9B). Asynchronously growing 293A cells were transfected with a reporter vector carrying the luciferase gene under the control of the basally active thymidine kinase promoter linked to five tandem copies of the GAL4 upstream activation sequence. As previously described (10, 14, 17), cotransfection of this reporter plasmid with GAL4bd alone led to an approximately two-fold activation of transcription above basal level (Fig. 9D, column 2). In contrast, cotransfection of GAL4bd-Gro/TLE1 led to a repression of both activated and basal transcription (Fig. 9C, column 2). The repression ability of GAL4bd-Gro/TLE1 was enhanced in a statistically significant manner when cells were incubated in the presence of the cdc2 inhibitor roscovitine (Fig. 9C, column 3). Roscovitine had no repressive effect on transcription in the presence of GAL4bd alone (Fig. 9D, column 3). More importantly, control experiments with the protein HDAC4, which can mediate transcriptional repression in a Gro/TLE-independent manner (46), showed that roscovitine did not enhance repression mediated by GAL4bd-HDAC4 (Fig. 9C, columns 4 and 5). Similar results were obtained in HeLa cells (Figs. 9E and F), and when
olomoucine was used to inhibit cdc2 activity (not shown). Together, these results show that inhibition of cdc2 activity results in a potentiation of the transcription repression ability of Gro/TLE1.
DISCUSSION

Involvement of cdc2 in Gro/TLE Phosphorylation - Gro/TLE family members are phosphorylated proteins that can associate with a variety of transcription factors and either provide a corepressor activity to dedicated transcriptional repressors (12, 13, 16, 23) or convert transactivators into repressors (53, 54). A number of observations suggest that phosphorylation mechanisms are involved in the regulation of the functions of Gro/TLEs. First, these proteins contain evolutionarily conserved consensus phosphorylation sites for a number of kinases (38). Second, the phosphorylation state of Gro/TLEs changes as a function of cell differentiation in neural and non-neural tissues (17, 36, 39). Third, they become phosphorylated in response to interaction with DNA-binding partners like Hes1 and RUNX1 (17), or Pax5 (22). Phosphorylation induced by Hes1 involves the activity of protein kinase CK2 and is correlated with a strong interaction of Gro/TLEs with the nuclear compartment (17). Fourth, phosphorylation by protein kinase CK2 promotes Gro/TLE-mediated transcriptional repression (17). Together, these findings point to important roles for phosphorylation mechanisms in Gro/TLE functions.

In this study, we have examined the regulation of Gro/TLE activity by cell cycle-regulated phosphorylation events. We have found that Gro/TLEs become hyperphosphorylated when cdc2 is conditionally activated by okadaic acid, a selective inhibitor of protein phosphatase 2A (43, 47, 49). We tested concentrations of okadaic acid that were shown to promote entry into mitosis and activate cdc2 but have no effect on the activities of numerous other kinases including the G1/S cell cycle-dependent kinases cdk2, cdk4, and cdk6 (47, 49), protein kinase CK1 and CK2, glycogen synthase kinase-3, protein kinase C, and others (43, 47, 49, 50). The okadaic
acid-induced phosphorylation of Gro/TLEs was blocked by treatment with pharmacological inhibitors of cdc2 like roscovitine and olomoucine; this effect mimicked the reversal of okadaic acid-induced hyperphosphorylation mediated by alkaline phosphatase treatment, indicating that cdc2 inhibitors reduce the phosphorylation state of Gro/TLE in vivo. Although these cdc2 inhibitors can also inhibit cdk2 and cdk5 (44, 45), these kinases are not known to be activated by okadaic acid and are not active at mitosis, further suggesting that cdc2 is involved in the okadaic acid-induced phosphorylation of Gro/TLEs. We have demonstrated further that Gro/TLEs are hyperphosphorylated in G2/M-arrested cells concomitant with activation of cdc2, and that treatment with cdc2 inhibitors decreases the phosphorylation of Gro/TLE proteins present in non-nuclear fractions isolated from G2/M cells. Taken together, these findings strongly suggest that cdc2 is involved in the phosphorylation of Gro/TLEs.

This possibility is supported further by our finding that purified cdc2-cyclin B can directly phosphorylate Gro/TLEs in vitro. The preferred phosphorylation site appears to be the CcN domain, originally named because it harbors possible phosphorylation sites for protein kinase CK2 and cdc2 adjacent to a Nuclear localization sequence (38). Importantly, we have demonstrated that a short deletion removing the sequence SSPRASPAHSPR from the CcN domain of Gro/TLE1 almost completely abolished the okadaic acid-induced hyperphosphorylation. Because this sequence contains at least one motif resembling the consensus cdc2 phosphorylation sequences, S/T-S/T-P-R/K or S/T-P-X-R/K (35), our results strongly suggest that cdc2 phosphorylates Gro/TLE1 at the CcN motif in response to okadaic acid treatment in vivo. This possibility is in agreement with previous studies showing that the presence of a CcN motif in other proteins is correlated with phosphorylation by cdc2, resulting in decreased nuclear association (55). Our studies have shown further that the amino-terminal half
of the SP domain, so named because of its abundance of Serine/Threonine and Proline residues (38), can also be phosphorylated by cdc2 in vitro. A deletion of roughly 50 amino acids that removes several phosphorylatable Serine/Threonine residues from this region of the SP domain was correlated with a modest but detectable decrease of the Gro/TLE hyperphosphorylation in response to okadaic acid, suggesting that this portion of Gro/TLE may also be phosphorylated in vivo. Future studies will be aimed at precisely determining which individual residues within the CcN and SP domains are phosphorylated by cdc2 in vivo.

Phosphorylation of Gro/TLE Proteins at Mitosis is Correlated with Reduced Nuclear Association – Previous immunocytochemical and biochemical studies have shown that Gro/TLEs are nuclearly localized in interphase cells (6, 17, 26). Moreover, the retention of Gro/TLE proteins in nuclear fractions obtained after biochemical cell fractionation was shown to result from their interaction with transcriptionally competent sites like chromatin and the nuclear matrix (6, 17, 26). Our present investigations have shown that hyperphosphorylated Gro/TLEs present at the G2/M transition are recovered preferentially in non-nuclear fractions upon subcellular fractionation. This behavior does not appear to result from a phosphorylation-induced nuclear export of Gro/TLEs because we have found that Gro/TLEs are localized to nuclei of G2/M-arrested cells by performing immunocytochemical studies (data not shown). Thus, based on the fact that immunocytochemical staining performed after fixation still detects a nuclear localization whereas biochemical fractionation performed without fixation reveals a loss of nuclear retention, we propose that phosphorylation events mediated by cdc2 weaken the association of Gro/TLE with nuclear components, resulting in reduced nuclear retention. More specifically, these phosphorylation events may negatively regulate the ability of Gro/TLEs to interact with components of chromatin and/or the nuclear matrix, thereby playing a negative
regulatory role in Gro/TLE functions. Such a model is consistent with the observation that mitotic phosphorylation events decrease the interaction with chromatin of another mammalian protein, termed HIRA, that is structurally related to Gro/TLEs and is also involved in transcriptional repression (42).

A Proposed Model for Cell Cycle-Dependent Regulation of Gro/TLE Activity – Since a reduced interaction with the nuclear compartment is expected to negatively affect the ability of Gro/TLEs to repress transcription, we tested whether the pharmacological inhibition of cdc2 activity would lead to a potentiation of Gro/TLE-mediated transcriptional repression. Our studies have shown that treatment of unsynchronized cultures (where only a fraction of cells are undergoing mitosis at any given time) with cdc2 inhibitors results in an increase in Gro/TLE-mediated repression. This was not a general effect because the same treatment did not result in a potentiation of the transcriptional repression mediated by HDAC4. This protein was chosen as a negative control because it was demonstrated that HDAC4 does not interact with Gro/TLEs (46). It seems unlikely that the enhancing effect on Gro/TLE-mediated repression derives from the inhibition of phosphorylation mechanisms occurring in interphase cells, where Gro/TLE are predominantly associated with the nuclear compartment and thus should be able to effectively mediate repression. Rather, we propose that cdc2-mediated phosphorylation events occurring in mitotic cells negatively regulate Gro/TLE activity, and that inhibition of such events enhances Gro/TLE-mediated transcriptional repression. The possibility that phosphorylation of Gro/TLEs at mitosis is a mechanism that contributes to the inactivation of these proteins during cell division is consistent with the demonstration that several other transcription factors are negatively regulated during mitosis as a result of phosphorylation events (56, 57). Given the ability of Gro/TLEs to form transcription complexes with a variety of DNA-binding proteins,
regulation of the nuclear interaction of the former may represent a general mechanism to control the functions of several transcription factors in mitotic cells.
ACKNOWLEDGMENTS

We thank Junaid Husain for his contribution to this study and Rita Lo for excellent technical assistance. We also thank X.-J. Yang, D.L. Barber, and A. Halupa for reagents. This work was supported by grants from the Canadian Institutes of Health Research (50%) and the Cancer Research Society Inc. (50%) to S.S.
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FOOTNOTES

1 The abbreviations used are: bHLH, basic helix loop helix; cdc2, p34\textsuperscript{cdc2}; CcN, protein kinase CK2, cdc2, nuclear localization sequence; GAL4bd, DNA-binding domain of GAL4; GP, Glycine/proline-rich; Gro, Groucho; GST, glutathione S-transferase; HDAC, histone deacetylase; Hes, Hairy and Enhancer of split; HMG, high-mobility group; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; Q, Glutamine-rich; SP, Serine/Proline-rich; TLE, transducin-like Enhancer of split; WDR, WD40 repeat.
FIGURE LEGENDS

Fig. 1. **Induction of Gro/TLE phosphorylation in response to okadaic acid treatment.** A-C, logarithmically growing ROS17/2.8 cells were either not treated (*lane 1*) or treated with the indicated amounts of okadaic acid (*Okadaic Ac.*, *lanes 5-7*) or dimethyl sulfoxide alone (*DMSO*, *lanes 2-4*) for 4 hours. Whole-cell lysates were prepared and equal amounts of proteins (~50 µg of protein/lane) were subjected to SDS-PAGE, transfer to nitrocellulose and Western blotting with antibodies (*Ab.*) against either Gro/TLE (*A, panTLE*), phosphorylation state-independent cdc2 (*B, anti-cdc2*), or inactive cdc2 phosphorylated at Tyr15 (*C, anti-phos. cdc2*). D, cells were either not treated (*lanes 1 and 2*) or treated (*lanes 3 and 4*) with okadaic acid, followed by preparation of cell lysates, incubation in the absence (*lanes 1 and 3*) or presence (*lanes 2 and 4*) of calf intestinal phosphatase (*CIP*), and Western blotting with panTLE antibodies. Okadaic acid treatment resulted in Gro/TLE hyperphosphorylation concomitant with cdc2 activation. Here and in succeeding figures, the positions of migration of *M_r* standards are indicated.
Inhibition of okadaic acid-induced phosphorylation of Gro/TLEs by pharmacological inhibitors of cdc2. Logarithmically growing ROS17/2.8 cells were either not treated (lanes 1 and 2) or treated with 500 nM okadaic acid (Okadaic Ac., lanes 3-6) in the absence (lanes 1 and 3) or presence (lanes 2 and 4-6) of the indicated concentrations of either olomoucine (A) or roscovitine (B). Whole-cell lysates were prepared and equal amounts of proteins were subjected to SDS-PAGE, transfer to nitrocellulose and Western blotting with panTLE monoclonal antibodies. Both olomoucine and roscovitine abolished the okadaic acid-induced phosphorylation of Gro/TLEs (long arrow points to hyperphosphorylated forms), and also led to Gro/TLE dephosphorylation in the absence of okadaic acid (short arrow).
Fig. 3. **Phosphorylation of Gro/TLE proteins by a purified cdc2-cyclin B complex.** Lysates from either Drosophila S2 (A and B) or human HeLa (C and D) cells were subjected to immunoprecipitation with anti-Gro monoclonal (A and B, lanes 1 and 2), control monoclonal (A and B, lane 3), anti-Gro/TLE1 polyclonal (C and D, lane 1), or control preimmune polyclonal (C and D, lane 2) antibodies (Ab.). Immunoprecipitates were extensively washed, followed by incubation with [γ-32P]-ATP in the absence (A and B, lane 1) or presence of purified cdc2-cyclin B (A and B, lanes 2 and 3; C and D, lanes 1 and 2). Samples were then subjected to SDS-PAGE, transfer to nitrocellulose and autoradiography (A and C) or Western blotting with anti-Gro (B) or panTLE (D) antibodies. Gro/TLE proteins were phosphorylated by cdc2 (A, lane 2; C, lane 1, long arrow). Cyclin B was also phosphorylated by cdc2 (A and C, open arrow). (B and D) Western blotting analysis confirmed the presence of immunoprecipitated Gro/TLE proteins.
Fig. 4. **Phosphorylation of the CcN and SP domains of Gro/TLE by a purified cdc2-cyclin B complex.**  
A, schematic representation of the domain structure of Gro/TLE proteins, as originally described in (38). The indicated numbers define the boundaries between domains; residues 269-374 encompass the “SP-N” region.  
B and C, the indicated GST fusion proteins were purified and subjected to *in vitro* phosphorylation assays in the presence (*lanes* 1, 3, 5, 7, 9, and 11) or absence (*lanes* 2, 4, 6, 8, 10, and 12) of purified cdc2-cyclin B, followed by SDS-PAGE, transfer to nitrocellulose and autoradiography (*C*) or Western blotting with anti-GST antibody (*Ab.*) (*B*). Both the CcN and SP domains were phosphorylated by cdc2 (*C, lanes* 7 and 9).
Fig. 5. Effect of deletion of amino acids 258-335 on the in vitro phosphorylation of Gro/TLE1 by a purified cdc2-cyclin B complex. Either purified histone H1 or the indicated GST fusion proteins were subjected to in vitro phosphorylation assays in the presence (lanes 1, 3, and 5) or absence (lanes 2, 4, and 6) of purified cdc2-cyclin B, followed by SDS-PAGE, transfer to nitrocellulose and autoradiography (B and D) or Western blotting with anti-GST antibody (Ab.) (A and C). The amino-terminal half of the SP domain (SP-N) was phosphorylated by cdc2 (B, lane 5), as was the combined CcN/SP-N region (D, lane 3). Deletion of amino acids 258-335 reduced phosphorylation to almost background levels. The GST-SP(N) fusion protein was unstable in bacterial cells. Empty lanes separated certain sets of samples to prevent possible spill over artifacts.
Fig. 6. **Effect of selected deletions on the okadaic acid-induced phosphorylation of Gro/TLE.** A, the sequence of the carboxy-terminal part of the CcN domain and the amino-terminal region of the SP domain of Gro/TLE1 (amino acids 256-336) is shown. The region containing consensus sites for phosphorylation by cdc2 in the CcN domain is shown in boldface and enclosed in a box, and Ser/Thr-Pro motifs in the SP domain are shown in boldface. B, 293 cells were transfected with fusion proteins of GAL4bd and either wild type Gro/TLE1 (WT; *lanes* 4-6) or Gro/TLE1(Δ258-268) (*lanes* 1-3). Twenty-four hours later, cells were either treated (*lanes* 2, 3, 5, and 6) or not treated (*lanes* 1 and 4) with 500 nM okadaic acid in the absence (*lanes* 1, 2, 4, and 5) or presence (*lanes* 3 and 6) of 100 µM olomoucine. Whole-cell lysates were prepared and subjected to Western blotting with panTLE antibodies. C, similar experiments were performed to determine the effect of okadaic acid treatment (*lanes* 2, 4, and 6) on the phosphorylation state of either wild type Gro/TLE1 (*lanes* 1 and 2), Gro/TLE1(Δ285-335) (*lanes* 3 and 4), or Gro/TLE1(Δ258-335) (*lanes* 5 and 6).
Fig. 7. **Phosphorylation of Gro/TLE proteins during the cell cycle.** Actively growing HeLa cells were treated with either hydroxyurea to enrich for G_{1}/S-arrested cells (*lanes* 1 and 2) or nocodazole to force arrest at the G_{2}/M transition (*lanes* 3 and 4), followed by metabolic labeling with $[^{32}\text{P}]\text{Pi}$. Whole-cell extracts were then subjected to immunoprecipitation with anti-Gro/TLE1 (*lanes* 2 and 4) or preimmune (*lanes* 1 and 3) serum, followed by SDS-PAGE, and autoradiography. Phosphorylated Gro/TLE forms of ~90 kDa were observed in both G_{1}/S- and G_{2}/M-enriched cultures (short arrow), while hyperphosphorylated species of ~95 kDa were preferentially observed in G_{2}/M cells (long arrow). As previously observed (36), a ~70-kDa phosphoprotein coimmunoprecipitated with Gro/TLE1 from both G_{1}/S- and G_{2}/M-arrested cells.
Fig. 8. **Reduced nuclear interaction of hyperphosphorylated Gro/TLE proteins at mitosis.**

HeLa cells were cultured in the presence of either hydroxyurea (*lanes 1 and 2*) or nocodazole (*lanes 3 and 4*), followed by preparation of non-nuclear (*Post Nucl. Sup.*, *lanes 1 and 3*) or nuclear (*Nucl. Extract.*, *lanes 2 and 4*) fractions. Samples were subjected to SDS-PAGE on either 10% (*A*), 13% (*B-D*), or 15% (*E*) gels, followed by transfer to nitrocellulose and Western blotting with antibodies against either TLE (*A*), inactive cdc2 phosphorylated at Tyr15 (*B*), phosphorylation-state independent cdc2 (*C*), HDAC2 (*D*), or histone H3 (*E*). *Lane 1* contained ~60 µg of protein; *lanes 2 and 3* contained ~50 µg of protein; *lane 4* contained ~40 µg of protein.

(*A*) Gro/TLE proteins were predominantly associated with the nuclear compartment in G1/S cells; in contrast, hyperphosphorylated Gro/TLEs present in G2/M cells (arrow) were preferentially found in the non-nuclear fraction. (*B and C*) cdc2 was observed mostly in an inactive state in the post-nuclear supernatant of hydroxyurea-treated cells and became activated at mitosis (some active cdc2 was also observed in the nuclear fraction of hydroxyurea-treated cells).
Fig. 9. Enhancement of Gro/TLE-mediated transcriptional repression by inhibition of cdc2 activity. A, expression of GAL4bd-Gro/TLE1. 293A cells were transfected with GAL4bd-Gro/TLE1 and cultured in the presence of either hydroxyurea (lanes 1 and 2) or nocodazole (lanes 3 and 4), followed by preparation of non-nuclear (Post Nucl. Sup., lanes 1 and 3) or nuclear (Nucl. Extract., lanes 2 and 4) fractions. Western blotting analysis revealed that GAL4bd-Gro/TLE1 was preferentially associated with the nuclear fraction in hydroxyurea-treated cells (lane 2), whereas it was recovered mostly in the non-nuclear fraction in G2/M cells (lane 3). Increased phosphorylation of GAL4bd-Gro/TLE1 at mitosis was not well visible in the particular gel shown here. B, nocodazole-treated cells arrested at the G2/M transition were not treated (lane 1) or treated (lane 2) with roscovitine, followed by preparation of non-nuclear fractions and Western blotting analysis with anti-GAL4bd antibody. The mobility of GAL4bd-Gro/TLE1 increased as a result of roscovitine treatment. C-F, transient transfection/transcription assays. 293A (C and D) or HeLa (E and F) cells were transfected with the reporter plasmid p5xGAL4UAS-tk-luciferase (0.5 µg) alone or in the presence of GAL4bd, GAL4bd-Gro/TLE1, or GAL4bd-HDAC4. A plasmid encoding β-galactosidase was co-transfected in each case to normalize the assays. Twenty-four hours later, cells were incubated in the absence or presence of roscovitine (10 µM), as indicated, and then cultured for an additional twenty-four hours. C and E, fold repression by either GAL4bd-Gro/TLE1 or GAL4bd-HDAC4 is shown as relative luciferase activity measured with GAL4bd alone divided by the relative activity in the presence of GAL4bd-Gro/TLE1 or GAL4bd-HDAC4. D and F, fold activation by GAL4bd alone is shown as relative luciferase activity measured with GAL4bd alone divided by the relative activity in the presence of empty expression vector. Values represent means ± standard deviation.
of at least 4 experiments performed in duplicates. The ability of Gro/TLE1 to mediate transcriptional repression was increased by roscovitine (*p, <0.02; **p, <0.005).
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 8

Hydroxyurea: + + - -
Nocodazole: - - + +
Post Nuc. Sup.: + - + -
Nucl. Extract: - + - +

A 116 97
B 31
C 31
D 66
E 21 14

WB: panTLE Ab.
WB: anti-phos. cdc2 Ab.
WB: anti-cdc2 Ab.
WB: anti-HDAC2 Ab.
WB: anti-H3 Ab.
Figure 9
A role for cell cycle-regulated phosphorylation in groucho-mediated transcriptional repression
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J. Biol. Chem. published online October 22, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M111660200

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