Phosphorylation of Serine 239 of Groucho/TLE1 by Protein Kinase CK2 Is Important for Inhibition of Neuronal Differentiation

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Transcriptional corepressors of the Groucho (Gro)/TLE family play important roles during a variety of developmental pathways, including neuronal differentiation. In particular, they act as negative regulators of neurogenesis, together with Hairy/Enhancer of split (Hes) DNA-binding proteins. The interaction with Hes1 leads to Gro/TLE hyperphosphorylation and increased transcription repression activity in mammalian cells, but the underlying molecular mechanisms are poorly characterized. We now show that Gro/TLE1 is phosphorylated in vivo by protein kinase CK2. This phosphorylation occurs at serine 239 within the conserved CcN domain present in all Gro/TLE family members. Mutation of serine 239 into alanine decreases Hes1-induced hyperphosphorylation of Gro/TLE1 and also reduces its nuclear association and transcription repression activity. We demonstrate further that Gro/TLE1 inhibits the transition of cortical neural progenitors into neurons and that its antineurogenic activity is inhibited by a serine-239-alanine mutation but not by a serine-239-glutamate mutation. These results suggest that CK2 phosphorylation of serine 239 of Gro/TLE1 is essential for its function during neuronal differentiation.

The Drosophila transcription factor Groucho (Gro) and its mammalian homologs the transducin-like Enhancer of split (TLE) proteins play fundamental roles during a wide range of developmental pathways. The archetypal Drosophila gro gene, which resides within the Enhancer of split locus, was originally identified by a viable mutation causing a phenotype with excessive sensory bristles above the eye, the result of impaired inhibition of neurogenesis. Gro is also required for a variety of other developmental events, including embryonic segmentation, dorsoventral patterning, and sex determination (7). Similarly, vertebrate Gro/TLE proteins are expressed in a variety of tissues and are involved in several developmental processes, including neural development (17, 21, 34), pituitary development (2, 5, 9), and eye formation (17). Thus, in both invertebrates and vertebrates, Gro/TLE family members play roles in the regulation of numerous developmental programs.

Gro/TLEs are transcriptional corepressors that lack DNA-binding activity of their own but are instead recruited to specific gene regulatory sequences via interaction with a number of different DNA-binding transcription factors. These include a family of related basic-helix-loop-helix proteins encoded by the hairy and Enhancer of split (Hes) genes of Drosophila (25) and their related vertebrate counterparts (13, 20, 22). A number of other transcription factors act as DNA-binding partners of Gro/TLEs, including Runt homology domain proteins (18, 19, 32), homedomain factors containing the engrailed homology region 1 motif (16, 21), paired domain proteins (4, 10), Tcf/Lef-related HMG box factors (18, 27), and winged helix domain proteins (35). As a result of these interactions, Gro/TLEs become recruited to selected target genes in context-dependent manners. Upon their recruitment to DNA, they are believed to mediate transcriptional repression by at least two mechanisms. One is thought to involve interactions with both histones and histone deacetylases (HDACs) resulting in the modification of the histone acetylation state (6, 8, 24). The other is hypothesized to involve inhibitory interactions with components of the basal transcriptional machinery (36).

The mechanisms that regulate Gro/TLE activities during cell differentiation are poorly defined. Gro/TLE1 becomes increasingly phosphorylated as a function of neuronal differentiation (14, 22). A change to a hyperphosphorylated state was also observed after the interaction of Gro/TLE with Hes1, one of its transcriptional cofactors during neuronal development (22). These observations suggested that changes in Gro/TLE1 phosphorylation that occur in response to cofactor binding might play roles in the regulation of the neural functions of Gro/TLE. Here we provide the first demonstration that protein kinase CK2 phosphorylates Gro/TLE1 at S239 in vivo. Phosphorylation of S239 is critical for cofactor-activated phosphorylation (CAP) that Gro/TLE1 undergoes in response to Hes1 binding. We also provide evidence that mutation of S239 into alanine decreases both the nuclear association and the transcription repression activity of Gro/TLE1. Finally, we demonstrate that Gro/TLE1 inhibits the differentiation of cortical neural progenitor cells into neurons and that phosphorylation of S239 is required for this function. Together, these results demonstrate that Gro/TLE1 is a physiological substrate of CK2 and provide the first characterization of events involved in the regulation of Gro/TLE activity during neuronal differentiation.

MATERIALS AND METHODS

Plasmids. Construct pCMV2-FLAG-Gro/TLE1 was generated by digesting pBluescript-Gro/TLE1 with BanII, followed by the removal of protruding ends with T4 DNA polymerase and recovery of an ~1.6-kb fragment encoding the N-terminal region. This fragment was subcloned into pCMV2-FLAG digested with EcoRV. The remaining portion of the Gro/TLE1 cDNA was then subcloned as a SmaI restriction fragment. For analysis of Gro/TLE1 point mutants, the plasmids pCMV2-FLAG-Gro/TLE1(S239A), pCMV2-FLAG-Gro/
branes were subjected to Western blotting with anti-GST antibodies. Alterna-
ferase (GST) and individual Gro/TLE domains from bacteria and in vitro phos-
(Pierce), and retained antibodies were eluted and concentrated in a Cen-
mids pEBG-Hes1, pEBG-Hes1(TLE1(S239A/S253A), respectively, followed by subcloning into pGEX2. Plas-
ry amplification of the region of interest from pCMV2-Gro/TLE1 (S239A), pCMV2-Gro/TLE1(S239A/S253A) or pCMV2-Gro/TLE1(S239A/S253A), respectively, followed by subcloning into pGEX2. Plas-
mains pEBG-Hes1, pEBG-Hes1(WRPW), pEGFP. pCMV2-FLAG-Hes1, pCMV5-Runc2, and pG052E-Luc were described (12, 19, 20).

Antibody production. An 11-amino-acid peptide containing residues 235 to 245 of human Gro/TLE1 with S239 converted to phosphoserine was synthesized (SHYDpSDGDKSD; Biosource), conjugated to maleimide-coupled KLH (Pierce), and injected into rabbits by using Freund adjuvant. Sera were passed over an affinity column of the immunizing phosphopeptide coupled to SulfoLink (Pierce), and retained antibodies were eluted and concentrated in a Cen-

in vitro kinase assays. Purification of fusion proteins of glutathione S-trans-
ferase (GST) and individual Gro/TLE domains from bacteria and in vitro phos-
phorylation assays with 0.5 U of purified CK2 (New England Biolabs) were performed as described previously (22). After gel electrophoresis, proteins were transferred to nitrocellulose and exposed to film. After autoradiography, mem-
branes were subjected to Western blotting with anti-GST antibodies. Alterna-
tively, the products of kinase reactions were loaded onto SpinZyme phospho-
cellulose units (Pierce) and centrifuged at 3,000 × g for 30 s. The filters were then extensively washed with 75 mM sodium phosphoric acid and dried. Bound radioactivity was measured in a scintillation counter.

Pharmacological inhibition of CK2. The selective CK2 inhibitor 4,5,6,7-tetra-
bromobenzotiazolone (TBB) (28) was resuspended in dimethyl sulfoxide (DMSO). HEK293 cells were incubated in the presence of either DMSO alone or TBB at the concentrations indicated in the figure legends. Cells were then collected, and whole-cell lysates were prepared and subjected to Western blotting.

Cell culture, transfection, protein-protein interaction assays, biochemical fractionation, and Western blotting. HELa and HEK293 cells were cultured and, when appropriate, transfected by using the SuperFect reagent (Qiagen) (20, 22). Cell lysates were prepared and GST coprecipitation, immunoprecipitation, and Western blotting studies were performed as described previously (19, 22, 24). Antibodies used were anti-(pS239)Gro/TLE1, anti-GST, anti-FLAG, anti-HDAC2, anti-HDAC4 (Zymed), anti-phosphoserine (1:750, Abcam), and anti-HDAC2 (1:000; Santa Cruz), anti-FLAG (1:000; Sigma), and anti-histone H3 (1:000 [22, 23]). Postnuclear supernatant and chromatin-enriched fractions were prepared as described previously (22, 23). Immunoblots were scanned and analysis performed by using the NIH Image J program (rsb.info.nih.gov/ji).

Indirect immunofluorescence. Staged mouse embryos were collected, fixed, sectioned, and subjected to immunohistochemistry as described previously (34). All animal procedures followed the guidelines of the Canadian Council for Animal Care. HEK293 and cortical neocortical progenitor cells were cultured on four-well chamber slides (Nalgene Nunc, Int.) as described previously (12), fixed, and permeabilized in 0.1% IGEPAL in HEPES-buffered saline containing 4% paraformaldehyde. Primary antibodies for immunofluorescence were either rabbit anti-(pS239)Gro/TLE1, rabbit anti-Gro/TLE1, rat pan-TLE, mouse anti-
flav. Mouse anti-Ki67 (1:25; BD Pharmingen), and mouse anti-NeuN (1:50, Chemicon). Detection was as described previously (12).

Transcription assays. HEK293 cells were transiently transfected with the reporter plasmid pO6SE2-Luc in the absence or presence of plasmids encoding either Runx2, FLAG-Gro/TLE1, FLAG-Gro/TLE1(S239A) or FLAG-Gro/
TLE1(S239E) as described previously (19). In each case, 0.5 µg of pRSV-β-
galactosidase DNA was cotransfected to provide a means of normalizing the assays for transfection efficiency. Luciferase activity values were expressed as means ± the standard deviations.

Cortical neural progenitor cell culture, transfection, and analysis of neuronal differentiation. Primary cortical progenitor cell cultures were established from dorsal telencephalic cortices dissected from embryonic day 13.0 (E13.0) mouse embryos as described previously (12). Cells were seeded into four-well chamber slides (Nalgene Nunc) at 4 × 10^5 cells/well, coated with 0.1% poly-l-lysine and 0.2% laminin (BD Biosciences), cultured in Neurobasal medium supplemented with 1% N2, 2% B27, 0.5 mM glutamine, 1% penicillin-streptomycin (Invitro-
gen), and 40 ng of FGF2 (Collaborative Research)/ml. After 48 h in vitro, cells were transfected by using plasmids encoding either green fluorescent protein (GFP) alone (0.2 µg/well), or both GFP (0.2 µg/well) and Gro/TLE1 (full-length or point mutants) (0.8 µg/well). The total amount of DNA was adjusted to 1.0 µg and mixed with 50 µl of OptiMEM medium (Invitrogen), followed by incubation for 5 min. An equal volume of OptiMEM medium was mixed separately with Lipofectamine 2000 reagent (Invitrogen; 2 µg/µl of DNA) and then combined with the DNA mixture and incubated for 20 min. Half the medium was removed, and stored as a conditioned medium, and the DNA-Lipofectamine 2000 mix was then added dropdown to each well. After 24 h, the medium was removed and replaced with fresh medium that contained the conditioned medium. Approximately 2 days later, cells were fixed and subjected to double-label immunocyto-

RESULTS

In vitro phosphorylation of S239 and S253 of Gro/TLE1 by CK2. Gro/TLE proteins are phosphorylated in vitro by CK2 (22). To identify the sites of CK2 phosphorylation, individual Gro/TLE domains (Fig. 1A) (31) were isolated from bacteria as fusion proteins with GST (Fig. 1B and C) and subjected to in vitro kinase assays with purified CK2. The Gro/TLE1 CcN domain was efficiently phosphorylated (Fig. 1D, lane 7, and E, lane 3). In contrast, other domains were not appreciably phos-

FIG. 1. Mapping of Gro/TLE1 sites phosphorylated by CK2 in vitro. (A) Schematic representation of the domain structure of Gro/TLE1. Abbreviations: Q, N-terminal glutamine-rich domain; GP, glycine/proline-rich domain; CcN, domain containing a putative NLS and conserved phosphorylation sites; SP, serine/proline-rich domain; WDR, WD40 repeat domain. (B to E) The indicated fusion proteins of GST and individual Gro/TLE domains were isolated from bacteria and subjected to in vitro kinase assays in the absence or presence of purified CK2 as shown. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transfer to nitrocellulose, autoradiography (D and E) and Western blotting (WB) with anti-GST antibodies (B and C) were performed. (F) Comparison of the sequence surrounding S239 and S253 of Gro/TLE1 with the same region of other Gro/TLE family members (H, human; M, mouse; D, Drosophila; C, C. elegans). Boxed and shaded residues define the consensus CK2 phosphorylation sequence SDX(D/E); boxed residues define the consensus CK2 phosphorylation site (S/T)N(E/D)D. (G) The indicated fusion proteins of GST and either wild-type or mutated forms of the CcN domain of Gro/TLE1 were subjected to in vitro kinase assays in the absence or presence of purified CK2 as shown, followed by autoradiography (G) and Western blotting with anti-GST antibodies (H). (I) Quantification of amount of phosphate incorporated into the indicated fusion proteins of GST and either wild-type or mutated forms of the CcN domain of Gro/TLE1 after in vitro phosphorylation with purified CK2 (n = 3). Here and in succeeding figures, the positions of size standards are indicated in kilodaltons.
Although the reasons for this difference remain to be determined, Western blot analysis with an antibody directed against the CcN domain of Gro/TLE1 confirmed that all of the four fusion proteins corresponded to bona fide Gro/TLE1 (data not shown). Together, these results strongly suggest that both S239 and S253 of Gro/TLE1 are phosphorylated by CK2 in vitro.

**In vivo phosphorylation of S239 of Gro/TLE1 by CK2.** We focused on the Gro/TLE1 CK2 phosphorylation sequence SDGD, which is conserved among all mammalian Gro/TLE proteins. To determine whether S239 of Gro/TLE1 is phosphorylated in vivo, we generated antibodies against a peptide from Gro/TLE1 phosphorylated at this residue (Fig. 2A). The affinity-purified anti-(pS239)Gro/TLE1 antibody recognized neither bacterially purified GST nor a fusion protein of GST and the Gro/TLE1 CcN domain (Fig. 2C, lanes 1 to 4). In contrast, the antibody reacted strongly with the same GST-CcN fusion protein when this was preincubated in vitro by purified CK2 (Fig. 2B and C, lanes 5 and 6). All fusion proteins were expressed at equivalent levels (Fig. 2D). These findings show that anti-(pS239)Gro/TLE1 is specific to the Gro/TLE1 CcN domain phosphorylated at S239.

To demonstrate that anti-(pS239)Gro/TLE1 recognized full-length Gro/TLE1 phosphorylated at S239, HEK293 cells were transfected with FLAG epitope-tagged forms of either wild-type Gro/TLE1 or a Gro/TLE1(S239A) mutant. Immunoprecipitation of these proteins with an anti-FLAG antibody (Fig. 2F) followed by Western blotting with anti-(pS239)Gro/TLE1 revealed that the latter reacted only with wild-type Gro/TLE1 (Fig. 2E, lane 2) and not with Gro/TLE1(S239A) (Fig. 2E, lane 3). We next tested whether anti-(pS239)Gro/TLE1 recognized endogenous Gro/TLE1 present in HEK293 cells. Immunoprecipitation with either preimmune serum, a previously characterized anti-Gro/TLE1 antibody (14, 33, 34), or anti-(pS239)Gro/TLE1, followed by Western blotting with a monoclonal antibody (panTLE) that recognizes all Gro/TLE proteins (22, 23, 31), revealed that anti-(pS239)Gro/TLE1 immunoprecipitated bona fide Gro/TLE (Fig. 2G, lane 4). We demonstrated further that the anti-(pS239)Gro/TLE1 immunoreactivity was specific to a single band that was abolished in the presence of the phosphorylated peptide used for immunization but not its unphosphorylated equivalent (Fig. 2H).

These peptides had no effects on the immunoreactivity decorated by the non-phospho-specific Gro/TLE1 antibody (Fig. 2I). Immunocytochemical studies showed further that anti-(pS239)Gro/TLE1 stained the nuclei of untransfected HEK293 cells (Fig. 2J), a finding consistent with the previously demonstrated nuclear localization of Gro/TLE1 (33, 34). The anti-(pS239)Gro/TLE1 immunoreactivity was abolished by the presence of excess phosphorylated peptide (Fig. 2K) but not unphosphorylated peptide (Fig. 2L). Taken together, these findings show that Gro/TLE1 is phosphorylated at S239 in vivo. It is important to stress that because the sequence surrounding S239 is highly conserved among mammalian Gro/TLE family members (Fig. 1F), it is possible that the anti-(pS239)Gro/TLE1 antibody may also react with other Gro/TLE proteins if these are phosphorylated at positions equivalent to S239. In potential agreement with this, we found that anti-(pS239)Gro/TLE1 reacted with Gro/TLE proteins immunoprecipitated with anti-Gro/TLE3 or anti-Gro/TLE4 antibodies (data not shown). Although the interpretation of those results is complicated by the demonstrated ability of Gro/TLEs to heterodimerize with each other (7, 13), which may cause the coprecipitation of Gro/TLE1 with other family members, these observations suggest that the anti-(pS239)Gro/TLE1 antibody might provide a general means to monitor the phosphorylation of Gro/TLE proteins at residues equivalent to S239 of Gro/TLE1.

We next determined whether CK2 was involved in the phosphorylation of S239 in vivo. HEK293 cells were cultured in the absence or presence of the selective CK2 inhibitor TBB, a cell-permeable compound that was shown to be specific for CK2 against a panel of more than 30 other protein kinases (28). Because disruption of CK2 activity is lethal in both embryonic and cultured cells (3), we performed these assays under conditions of only partial CK2 inhibition to preserve cell viability (28, 29; data not shown). Lysates from cells treated with TBB or DMSO alone were subjected to Western blot analysis with anti-(pS239)Gro/TLE1. Exposure to TBB resulted in a significant decrease of anti-(pS239)Gro/TLE1 immunoreactivity (Fig. 3A and E, lanes 2 and 4) without decreasing the immunoreactivity detected with either the non-phospho-specific Gro/TLE1 antibody (Fig. 3B and E, lanes 2 and 4) or the panTLE antibody (Fig. 3C). Neither did TBB cause obvious changes in the immunoreactive profile decorated by an anti-phosphoserine antibody (Fig. 3D), in agreement with the demonstrated specificity of TBB for CK2 (28). Thus, TBB caused a specific decrease in the phosphorylation of S239 without affecting the expression levels of Gro/TLEs. These combined results show that Gro/TLE1 is phosphorylated in vivo by CK2 at S239.

**Importance of S239 for the nuclear association of Gro/TLE1.** S239 is located 11 amino acids C-terminal to the putative nuclear localization sequence (NLS) of Gro/TLE1 and 20 residues N-terminal to a stretch of possible phosphorylation sites for cdc2 kinase (22, 31). These adjacent CK2 and cdc2 phosphorylation sequences, together with the NLS, define the Gro/TLE CcN motif. The CcN motif has been identified in a number of nuclear proteins including the simian virus 40 (SV40) large tumor (T) antigen, nucleoplasmin, and interferon induced factor 16 (IFI 16) (reference 1 and references therein). In the cases tested, phosphorylation of the CcN motif by CK2 enhances nuclear accumulation, and this is believed to occur through the strengthening of intranuclear interactions with as-yet-uncharacterized components (1, 11). To determine whether phosphorylation of S239 was involved in the nuclear...
association of Gro/TLE1, we first tested whether mutation of this residue would affect its nuclear translocation. Immunocytochemical analysis of HEK293 cells transfected with either wild-type Gro/TLE1 or its S239A, S253A, or S239A/S253A derivatives revealed that all of these proteins localized to the nucleus (Fig. 4A to D). Thus, neither S239 nor S253 appears to be required for the nuclear translocation of Gro/TLE1.

Next, we examined whether S239 was important for events that stabilize the nuclear association of Gro/TLE1 after its nuclear localization. The nuclear interaction of wild-type Gro/TLE1 and Gro/TLE1(S239A) was compared through a previously described subcellular fractionation assay that measures the retention of these proteins in the nuclear compartment versus their release into a postnuclear supernatant (14, 23). Transfected cells were fractionated into non-nuclear and chromatin-enriched fractions. Compared to wild-type Gro/TLE1, the introduction of the S239A mutation caused a significant decrease in the ratio between the Gro/TLE1 immunoreactivity in the chromatin-enriched fraction and the immunoreactivity in the non-nuclear fraction (Fig. 4E and H). Reprobing of the

FIG. 2. Specificity of anti-(pS239)Gro/TLE1 antibody. (A) Sequence of the region of the CcN domain of Gro/TLE1 encompassing S239. The phosphopeptide used for immunization is indicated on top. (B to D) Increasing amounts of GST (lanes 1 and 2), GST-CcN fusion protein (lanes 3 and 4), GST-CcN that had been previously incubated with purified CK2 (lanes 5 and 6) were subjected to SDS-PAGE, transfer to nitrocellulose, autoradiography (B), and Western blotting with either anti-(pS239)Gro/TLE1 (C) or anti-GST (D) antibodies. (E and F) HEK293 cells were transfected with the indicated FLAG-tagged proteins, followed by immunoprecipitation with anti-FLAG antibody and Western blotting with either anti-(pS239)Gro/TLE1 (E) or anti-FLAG (F) antibodies. (G) Lysates from untransfected HEK293 cells were subjected to immunoprecipitation (IP) with either preimmune serum (lane 2), a previously characterized anti-Gro/TLE1 antibody (lane 3), or anti-(pS239)Gro/TLE1 (lane 4). The immunoprecipitates (lanes 2 to 4), together with 1/10 of the amount of input lysate used in each immunoprecipitation (lane 1), were subjected to Western blotting with panTLE monoclonal antibodies. An open arrow indicates the immunoglobulin heavy chain. (H and I) Lysates from untransfected HEK293 cells were subjected to Western blotting with either anti-(pS239)Gro/TLE1 (H) or anti-Gro/TLE1 (I) antibodies in the absence (lane 1) or presence of the indicated peptides (lanes 2 and 3). (J to L) Indirect immunofluorescence analysis of untransfected HEK293 cells with anti-(pS239)Gro/TLE1 antibody in the absence (J) or presence of the indicated amount (per well) of the phosphorylated peptide used for immunization (K) or its unphosphorylated version (L). (J′ to L′) Hoechst staining is shown.
same samples with antibodies against HDAC2 or histone H3 showed that this change was specific to Gro/TLE1(S239A) (Fig. 4F and G). Taken together with previous studies showing that Gro/TLEs interact with both chromatin and the nuclear matrix (15, 24), these results suggest that mutation of S239 does not perturb the nuclear translocation of Gro/TLE1 but may weaken its interaction with intranuclear structures, causing a decreased nuclear retention.

Involvement of S239 in Hes1-activated hyperphosphorylation of Gro/TLE1. We next investigated whether phosphorylation of the CcN domain of Gro/TLE1 by CK2 was important for the CAP that Gro/TLE1 undergoes after interaction with its transcriptional partner Hes1 (22). Wild-type and mutated Gro/TLE1 proteins were expressed in HEK293 cells in the absence or presence of cotransfected Hes1. As previously shown (22), Hes1 induced a significant CAP of Gro/TLE1 containing CK2-mediated phosphorylation of the CcN domain in Gro/TLE1(Fig. 5A), blocked the Hes1-mediated CAP of Gro/TLE1 (Fig. 5I). Taken together, these observations strongly suggest that phosphorylation of S239 takes place independently of Hes1 binding but is important for additional phosphorylation events that are activated by Hes1 binding and result in the CAP of Gro/TLE1. Such events may directly target the region deleted in Gro/TLE1(S239A) shown (22), Hes1 induced a significant CAP of Gro/TLE1 even in the absence of cotransfected Hes1 (Fig. 2H [see also Fig. 5G, lane 1]). Moreover, a small deletion removing 50 amino acids C-terminal to the CcN motif of Hes1 (Fig. 5E, lane 6) blocked the Hes1-mediated CAP of Gro/TLE1 (Fig. 5H, lanes 3 and 4) without perturbing the Gro/TLE1: Hes1 interaction (Fig. 5I). Taken together, these observations strongly suggest that phosphorylation of S239 takes place independent of Hes1 binding but is important for additional phosphorylation events that are activated by Hes1 binding and result in the CAP of Gro/TLE1. Such events may directly target the region deleted in Gro/TLE1(D285-335), which contains an abundance of serine and threonine residues. Alternatively, this region may be involved in conformational or protein-protein interaction mechanisms required for the Hes1-mediated CAP of Gro/TLE1.

Importance of S239 for Gro/TLE1-mediated transcriptional repression. Based on these results and the previous demonstration that inhibition of CK2 activity reduces the transcription repression ability of Gro/TLE (22), we next tested whether S239 was important for Gro/TLE1-mediated transcriptional repression. HEK293 cells were transfected with a reporter plasmid containing the luciferase reporter gene under the control of tandem DNA-binding sites for the Runt-related protein Runx2. Previous studies have shown that Runx2 activates transcription from these sites and that Gro/TLE can repress this transcription (32). In agreement with these results, we found that Runx2 induced a roughly 150-fold activation of reporter gene expression (designated as 100%) and wild-type Gro/TLE1 suppressed this activation (Fig. 6A, bars 1 to 4). In contrast, Gro/TLE1(S239A) did not repress Runx2-mediated
transactivation (Fig. 6A, bars 5 and 6) when expressed at equivalent levels (Fig. 6B). Runx2 interacted equally with Gro/TLE1 and Gro/TLE1(S239A) (data not shown). Importantly, a point mutation that introduced a negatively charged glutamate residue at position 239 [Gro/TLE1(S239E)] did not disrupt the transcription repression ability of Gro/TLE1 (Fig. 6A, bars 7 and 8). Together, these results strongly suggest that phosphorylation of S239 provides local negative charges that are important for the transcription repression function of Gro/TLE1.

Phosphorylation of S239 in both neural progenitors and differentiated neurons in the developing mouse cerebral cortex. Gro/TLE1 is dynamically expressed during neuronal differentiation in the developing cerebral cortex. In particular, it is initially expressed in the ventricular zone of the dorsal telencephalon, where undifferentiated neural progenitor cells are located, and then becomes downregulated in the intermediate zone containing newly born neurons. Its expression is again reactivated in more developmentally mature cells that have migrated to the cortical plate (33, 34) (Fig. 7B). Immunohistochemical analysis with anti-Gro/TLE1 and anti-(pS239)Gro/TLE1 antibodies revealed essentially overlapping patterns of expression in both the ventricular zone and the cortical plate of the dorsal telencephalon (Fig. 7B and C). Moreover, both of these antibodies produced a similar nuclear staining (Fig. 7D and E). Similarly, we found that most, if not all, of the panTLE-immunoreactive cells in the ventricular zone and cortical plate were also positive for anti-(pS239)Gro/TLE1 immunoreactivity (Fig. 7F to H). These findings suggest that S239 is constitutively phosphorylated in both cortical progenitors and differentiated neurons. To directly determine whether Gro/TLE1 is phosphorylated at S239 in dividing cortical progenitor cells, we performed double-labeling studies with antibodies against the proliferating cell marker protein, Ki67, and anti-(pS239)Gro/TLE1 antibodies. We found overlapping expression patterns in the cortical ventricular zone (Fig. 7I to K). Moreover, the anti-(pS239)Gro/TLE1 immunoreactivity overlapped with the expression of the neural progenitor cell marker protein, nestin, in primary cultures of cortical neural progenitor cells obtained from the dorsal telencephalon of E13.0 mouse embryos (Fig. 7L and M). Together, these findings show that Gro/TLE1 is expressed in proliferating neural progenitors of the murine telencephalon and that S239 is phosphorylated in these cells.

Inhibition of the neural progenitor-to-neuron transition by Gro/TLE1. The correlation between the downregulation of Gro/TLE1 expression and neuronal generation at the ventricular-intermediate zone border suggested that Gro/TLE1 might inhibit the transition of neural progenitors into neurons. To examine this, we established primary cultures of dividing cortical progenitor cells and monitored their proliferation and neuronal differentiation in the absence or presence of exogenous Gro/TLE1. Enhanced GFP was coexpressed to mark the transfected cells. Immunocytochemical analysis with antibodies to Ki67 revealed that, compared to GFP, exogenous Gro/TLE1 caused an increase in the number of mitotic, undifferentiated progenitor cells (Fig. 8A to C) and a decrease in the number of differentiated neurons identified through their morphology (not shown) and the expression of the neuron-specific protein NeuN (Fig. 8F, bars 1 and 2). These results show that Gro/TLE1 is phosphorylated at S239 in dividing cortical progenitor cells, we performed double-labeling studies with antibodies against the proliferating cell marker protein, Ki67, and anti-(pS239)Gro/TLE1 antibodies. We found overlapping expression patterns in the cortical ventricular zone (Fig. 7I to K). Moreover, the anti-(pS239)Gro/TLE1 immunoreactivity overlapped with the expression of the neural progenitor cell marker protein, nestin, in primary cultures of cortical neural progenitor cells obtained from the dorsal telencephalon of E13.0 mouse embryos (Fig. 7L and M). Together, these findings show that Gro/TLE1 is expressed in proliferating neural progenitors of the murine telencephalon and that S239 is phosphorylated in these cells.

**FIG. 4.** Involvement of S239 in the nuclear association of Gro/TLE1. (A to D) Immunocytochemical analysis of the expression of the indicated FLAG-tagged Gro/TLE1 proteins in HEK293 cells. Hoechst staining is shown in panels A to D. (E to H) HEK293 cells were transfected with the indicated Gro/TLE1 proteins and subjected to biochemical fractionation to obtain postnuclear supernatant (Pn.S.) and chromatin-enriched (Chrom.) fractions; Western blotting was then performed with either anti-FLAG (E), anti-HDAC2 (F), or anti-histone H3 (G) antibodies. (H) Quantitation of the results of three separate experiments such as the representative one depicted in panel E showing the following ratio: FLAG immunoreactivity in the chromatin-enriched fraction (Chrom. imm.)/FLAG immunoreactivity in the postnuclear supernatant fraction (Pn.S. imm.) for either wild-type Gro/TLE1 (left bar) or Gro/TLE1(S239A) (right bar). *, P < 0.05.
TLE1 inhibits the transition of cortical neural progenitor cells into postmitotic neurons.

**Involvement of S239 in the antineurogenic function of Gro/TLE1.** We next tested whether S239 was important for the ability of Gro/TLE1 to inhibit cortical neuronal differentiation. In contrast to wild-type Gro/TLE1, exogenous expression of the mutated protein Gro/TLE1(S239A) in cortical progenitor cells failed to cause a decrease in the number of differentiated neurons or an increase in undifferentiated cells compared to control conditions (Fig. 8E and F, bars 1 and 3). Gro/TLE1 and Gro/TLE1(S239A) were expressed at equivalent levels in the transfected cells (Fig. 8D). Thus, mutation of S239 to a nonphosphorylatable residue impairs the antineurogenic activity of Gro/TLE1. However, the Gro/TLE1(S239E) mutant inhibited neuronal differentiation like wild-type Gro/TLE1, indicating that replacement of S239 with a negatively charged amino acid that may mimic the presence of a phosphorylated residue did not perturb the Gro/TLE1 antineurogenic activity (Fig. 8E and F, bars 2 and 4). Examination of additional Gro/TLE1 mutants revealed that Gro/TLE1(S253A) was somewhat less effective than wild-type Gro/TLE1 in inhibiting neuronal differentiation (Fig. 8E and F, bars 2 and 5). This suggests that S253 may also contribute to the antineurogenic activity of Gro/TLE1, although its role may not be as important as that of S239. Gro/TLE1(S253E) displayed the same antineurogenic activity as wild-type Gro/TLE1 (Fig. 8E and F, bars 2 and 6). The double-mutant Gro/TLE1(S239A/S253A) displayed no antineurogenic activity (Fig. 8E and F, bars 2 and 7) even though it was properly expressed (Fig. 8D). Thus, this double-mutant behaved similar to the single mutant Gro/TLE1(S239A). In contrast, Gro/TLE1(S239E/S253E) inhibited neuronal differentiation like wild-type Gro/TLE1 (Fig. 8E and F, bars 2 and 8).
and F, bars 2 and 8). Taken together, these findings show that Gro/TLE1 inhibits cortical neurogenesis and that this function is dependent on the phosphorylation of S239.

**DISCUSSION**

Gro/TLE proteins are widely expressed transcriptional corepressors involved in the regulation of multiple genes. Little is known about the molecular mechanisms that modulate Gro/TLE activities. In the present study, we have obtained new evidence strongly suggesting that CK2 phosphorylation of S239 of Gro/TLE1 is important for its transcription repression activity and antineurogenic role during cortical neurogenesis. These results provide the first characterization of mechanisms involved in the regulation of Gro/TLE functions during neural development and possibly other developmental processes.

**Gro/TLE1 is phosphorylated by CK2 in vivo.** Through in vitro kinase assays and the use of a phospho-specific antibody, we have demonstrated for the first time that CK2 phosphorylates Gro/TLE1 at S239. This finding is consistent with the evolutionary conservation of this site within the CcN motifs of Drosophila Gro (231SDQD) and human Gro/TLEs (Gro/TLE1, 239SDGD; Gro/TLE2, 228SDED; Gro/TLE3, 240SDGD; Gro/TLE4, 238SDGE). In vitro kinase assays have suggested further that CK2 can also phosphorylate Gro/TLE1 at a second sequence, 253SNED, located 25 residues C terminal to the NLS. A similar CK2 consensus sequence is conserved in the CcN motif of the C. elegans Gro/TLE family member UNC-37 (182TNDD), human Gro/TLE3 (251SNED) and Gro/TLE4 (257SNED), but not Gro/TLE2 or Drosophila Gro. Although it remains to be determined whether the 257SNED site is phosphorylated by CK2 in vivo, the observation that UNC-37 contains a conserved TNDD, but not SDX(T/E), sequence C terminal to its NLS, suggests that S/TNE/DD motifs within the CcN domain of certain Gro/TLEs are also targets of CK2.

The conservation of at least one CK2 target site within the CcN domain of different Gro/TLE family members suggests that CK2-mediated phosphorylation of this region is an important event in the functions of all Gro/TLE proteins. Because CK2 is a ubiquitous and constitutively active protein kinase (reference 28 and references therein), it is likely that phosphorylation of Gro/TLE1 by CK2 is a constitutive event. In agreement with this, we have found that Gro/TLE1 becomes phosphorylated at S239 rapidly after its translation. Moreover, Gro/TLE1 is phosphorylated at S239 in virtually all cells examined. In particular, our results suggest that Gro/TLE1 is phosphorylated at S239 in both mitotic neural progenitor cells and postmitotic neurons in the developing cerebral cortex. These combined observations suggest that phosphorylation of S239 is not correlated with specific functions of Gro/TLE1 in either proliferating or differentiated cells but may instead represent a general regulatory event underlying its activity.

**Phosphorylation of S239 of Gro/TLE1 is important for nuclear interaction and transcriptional repression.** Insight into the role of CK2-mediated phosphorylation of Gro/TLE1 is provided by studies of other proteins, including SV40 T-antigen, nucleoplasmin, and IFI 16, that also contain CcN motifs that are phosphorylated at their resident CK2 sites (1; references 11 and references therein). Where examined, CK2 phosphorylation promotes the nuclear association of these proteins. Although it was originally hypothesized that CK2-mediated phosphorylation of the CcN motif increased the affinity of the NLS for the importin heterodimer, recent studies have shown that the region containing the CK2 phosphorylation site of the CcN motif of SV40 T-antigen does not make contacts with importins and phosphorylation does not increase the affinity of recognition by the NLS-recognizing importin heterodimer (11). Similarly, although CK2-mediated phosphorylation of the CcN motif of IFI 16 promotes its nuclear accumulation, it appears to do so independently of recognition by importins but rather through facilitating binding to nuclear components (1). In agreement with these observations, the present findings have shown that mutation of S239 into alanine does not prevent the nuclear localization of Gro/TLE1 but instead decreases its nuclear retention during subcellular fractionation. This observation is consistent with the previous demonstration that pharmacological inhibition of CK2 also decreases the nuclear retention of Gro/TLE1 (22). These combined results strongly suggest that CK2-mediated phosphorylation of the CcN domain of Gro/TLE1 is not required for nuclear translocation but instead promotes the nuclear interaction of Gro/TLE1.

Our studies have suggested at least one mechanism by which phosphorylation of the CcN domain contributes to the nuclear binding of Gro/TLE1. We have shown that both pharmacological inhibition of CK2 activity (22) and mutation of S239 into alanine result not only in a weakened nuclear interaction but also a reduced Hes1-mediated CAP of Gro/TLE1. These findings strongly suggest that the nuclear association of Gro/TLE1 is promoted by a hierarchical sequence of phosphorylation events in which Gro/TLE1 first becomes phosphorylated by CK2 within its CcN domain. This primary phosphorylation is...
FIG. 7. Expression of Gro/TLE1 during cortical neurogenesis. (A) Anatomical view of the forebrain of E13.0 mouse embryos; the boxed area corresponds to the region of the dorsal telencephalon shown in the studies depicted in panels B to K. (B to K) Immunohistochemical analysis of E13.0 mouse telencephalon with either anti-Gro/TLE1 (B and D), anti-(pS239)Gro/TLE1 (C, E, F, and I), panTLE (G), or anti-Ki67 (J) antibodies. (D and E) High-magnification views of the expression of Gro/TLE1 or (pS239)Gro/TLE1 in the ventricular zone. The dotted lines indicate the apical side of the ventricular zone abutting the telencephalic vesicle. (H) Combined (pS239)Gro/TLE1 and panTLE staining. (K) Combined (pS239)Gro/TLE1 and Ki67 staining. (L and M) Combined immunocytochemical analysis of cortical progenitor cell cultures with anti-(pS239)Gro/TLE1 (red; nuclear staining) and anti-nestin (green; non-nuclear staining) antibodies; Hoechst staining is shown in panel L.
not required for either nuclear translocation or interaction with Hes1 but is important for both the secondary phosphorylation of Gro/TLE1 that occurs during CAP and its nuclear retention. We propose that the constitutive, CK2-mediated phosphorylation of the CcN domain is required for subsequent, cofactor-regulated phosphorylation events that either directly or indirectly strengthen the interaction of Gro/TLE1 with yet-to-be-defined transcriptional or structural factors that stabilize its association with nuclei.

A corollary to this model is that inhibition of CK2 phosphorylation should reduce the ability of Gro/TLE1 to mediate transcriptional repression. In agreement with this, we have demonstrated that both pharmacological inhibition of CK2 activity (22) and mutation of S239 (the present study) weaken the ability of Gro/TLE to repress transcription. Moreover, mutation of S239 impairs the biological activity of Gro/TLE1 during neuronal differentiation, an effect that is consistent with a loss or reduction of transcription repression function (see below for further discussion). These combined observations suggest that a combination of constitutive and regulated posttranslational modifications establish a phosphorylation state that promotes intranuclear interactions that render Gro/TLE1 competent to mediate transcriptional repression.

Phosphorylation of S239 is important for Gro/TLE1 activity during cortical neurogenesis. The possible involvement of a hierarchy of phosphorylation reactions in the regulation of Gro/TLE1 activity is consistent with the observation that this protein becomes increasingly phosphorylated as a function of neuronal differentiation in primary cortical neural progenitors or P19 cells (14, 22). We have found that S239 is phosphory-
lated in both undifferentiated neural progenitors and differentiated neurons, suggesting that it is unlikely that these phosphorylation changes are due to a developmentally regulated phosphorylation of S239. However, we have demonstrated that mutation of S239 suppresses the ability of Gro/TLE1 to inhibit the transition of cortical neural progenitor cells into neurons. Gro/TLE1 is coexpressed with, and acts as a transcriptional corepressor for, different DNA-binding factors that inhibit and/or delay cortical neuronal differentiation and also promote the CAP of Gro/TLE1, including Hes1 and brain factor 1 (22, 30, 35). We therefore propose that S239 of Gro/TLE1 needs to be phosphorylated by CK2 in order to allow the developmentally regulated CAP of Gro/TLE1 that occurs during neurogenesis as a result of its interaction with such DNA-binding cofactors. In turn, the modification of Gro/TLE1 caused by the regulated CAP promotes its nuclear binding and transcription repression activity, which are required for its antineurogenic function. In this scenario, CK2 phosphorylation of Gro/TLE1 is a critical event for the regulation of its activity during neuronal development. Given the widespread expression of CK2, it is possible that similar mechanisms also modulate Gro/TLE activity outside of the nervous system.

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