The Winged-Helix Protein Brain Factor 1 Interacts with Groucho and Hes Proteins To Repress Transcription

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Brain factor 1 (BF-1) is a winged-helix transcriptional repressor that plays important roles in both progenitor cell differentiation and regional patterning in the mammalian telencephalon. The aim of this study was to elucidate the molecular mechanisms underlying BF-1 functions. It is shown here that BF-1 interacts in vivo with global transcriptional corepressors of the Groucho family and also associates with the histone deacetylase 1 protein. The ability of BF-1 to mediate transcriptional repression is promoted by Groucho and inhibited by the histone deacetylase inhibitor trichostatin A, suggesting that BF-1 recruits Groucho and histone deacetylase activities to repress transcription. Our studies also provide the first demonstration that Groucho mediates a specific interaction between BF-1 and the basic helix-loop-helix protein Hes1 and that BF-1 potentiates transcriptional repression by Hes1 in a Groucho-dependent manner. These findings suggest that Groucho participates in the transcriptional functions of BF-1 by acting as both a corepressor and an adapter between BF-1 and Hes1. Taken together with the demonstration that these proteins are coexpressed in telencephalic neural progenitor cells, these results also suggest that complexes of BF-1, Groucho, and Hes factors may be involved in the regulation of progenitor cell differentiation in the telencephalon.

In the vertebrate central nervous system (CNS), differentiated neuronal and glial cells derive from proliferating progenitors located in the ventricular zone of the neural tube. The mechanisms that regulate the commitment of these progenitor cells to the neuronal fate are under the control of either positive or negative regulators. Proteins that promote neuronal differentiation include a family of related DNA-binding proteins containing the basic helix-loop-helix (bHLH) motif. These factors, generally referred to as the proneural proteins (reviewed in reference 31), are transcriptional activators that promote the expression of genes that contribute to the regulatory cascade of events leading to the formation of postmitotic neurons (15, 20, 33, 36, 37).

Negative regulators of neuronal differentiation comprise a number of structurally distinct proteins that act together to antagonize the activities of the proneural proteins. Important members of this functional class include components of the Notch signaling pathway, like the transmembrane receptor Notch, extracellular ligands of Notch, and intracellular factors that mediate responses to Notch activation (reviewed in references 3 and 52). Notable among the latter are the bHLH DNA-binding proteins of the Hairy/Enhancer of split (Hes) family (1, 14, 26, 27, 39, 40) and the transcriptional corepressors of the Groucho/transducin-like Enhancer of split (TLE) family (11, 18, 32, 47, 55). Hes and Groucho/TLE proteins are thought to form transcription repression complexes that inhibit proneural gene activity in response to Notch activation (18, 23, 28, 40, 41). Within these complexes, Hes proteins provide a specific DNA-binding function while Groucho/TLEs provide a transcription repression function.

In contrast to the progress made in understanding the mechanisms that regulate neuronal determination, relatively little is known about the events that control the establishment of the correct temporal and spatial patterns of neuronal differentiation along the anteroposterior axis of the CNS. Recently, the discovery of a number of genes that are expressed in restricted patterns within the neural tube has provided ways to begin to investigate the mechanisms controlling regional differentiation in the CNS. In this regard, the winged-helix transcription factor brain factor 1 (BF-1) (48) (recently renamed Foxg1 [30]) was identified as a protein whose expression in the developing murine brain is restricted to the telencephalon and the nasal half of the retina and optic stalk. In these tissues, *BF-1* is expressed in both mitotic neural progenitor cells and postmitotic neurons (22, 48). A closely related protein, termed BF-2, is expressed in the immediately adjacent region, the rostral diencephalon (22). Targeted disruption of *BF-1* function by homologous recombination causes hypoplasia of the cerebral hemispheres in mouse embryos. This phenotype appears to be caused by the premature differentiation of neural progenitor cells, resulting in an early depletion of the progenitor cell population (24, 53). The forebrain of *BF-1*^{-/-} embryos also displays dorsoventral patterning defects, suggesting that BF-1 may be involved in the regulation of both progenitor cell differentiation and local patterning events (53). A role for BF-1 in regulating the transition of progenitor cells to postmitotic neurons in the forebrain is also suggested by the analysis of the *Xenopus* BF-1 homolog, XBF-1. Like its murine counterpart, XBF-1 is specifically expressed in precursor cells of anterior neural structures (5). Ectopic expression of high levels of XBF-1 in posterior neural plate cells inhibits neuronal differentiation (5), in agreement with the notion that BF-1 proteins may represent anterior-specific factors involved in the regulation of neuronal differentiation.

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Although little is presently known about the molecular mechanisms underlying BF-1 function, transient transfection studies have shown that BF-1 proteins can mediate transcriptional repression (7, 35). In this regard, several observations have raised the possibility that the repression functions of BF-1 may involve interactions with general transcriptional corepressors of the Groucho/TLE family. First, *BF-1* and *TLE* genes are coexpressed in neural progenitor cells of the mammalian telencephalon (11, 53–55), and at least one TLE family member, TLE1, is involved in the regulation of forebrain development in vivo (55). Second, TLE proteins interact with other factors containing the winged-helix motif, like hepatic nuclear factor 3 β (51). Third, studies of *Xenopus* embryos have shown that the phenotypes caused by ectopic expression of full-length XBF-1 can be phenocopied by fusion proteins of the DNA-binding domain of XBF-1 and the repression domain of the Engrailed protein (5). Similarly, the embryonic phenotypes caused by ectopic expression of the related *Xenopus* protein, XBF-2, can be phenocopied by fusion proteins of the XBF-2 DNA-binding domain and the repressor domain of either Engrailed or Hairy (38). These chimeric proteins are likely to function in association with Groucho/TLE proteins, since both the Engrailed and Hairy repression domains were shown to mediate interactions with Groucho/TLEs (14, 29, 43, 50). Taken together, these observations suggest that the recruitment of Groucho/TLE corepressors may be a mechanism normally utilized by BF-1 to repress transcription.

Here we describe experiments designed to test the possible involvement of TLE proteins in the transcriptional functions of murine BF-1. Our results are consistent with a model in which BF-1 recruits TLEs and histone deacetylases to repress transcription. Moreover, TLEs may act as adapters between BF-1 and the Hes family member Hes1. These findings suggest that BF-1, Hes1, and TLE proteins may form transcription repression complexes that may coordinate the regulation of cell cycle progression and cell differentiation during the development of the mammalian forebrain.

MATERIALS AND METHODS

Plasmids. The following is a summary of the names and origins of the constructs used in these studies. Additional information on cloning strategies and oligonucleotide primers used in PCR experiments is available upon request. Vent DNA polymerase was used, and PCR products were routinely sequenced before subcloning into the appropriate vectors. For expression of glutathione *S*-transferase (GST) fusion proteins in bacteria, the constructs pGEX2-TLE1(1–135) [Gln-rich region [Q domain] of TLE1], pGEX1-TLE1(290–461) [Ser/Pro-rich region [SP domain] of TLE1], pGEX3-TLE3(490–774) (tandem WD40 repeat [WDR] domain of TLE3), and pGEX1-Hes1(3–281) (the entire Hes1 sequence except for the first two amino acids) were generated as described previously (18, 19, 39). pGEX3-BF-1 was obtained by first using site-directed mutagenesis to create a *PvuII* site at the 5' end of the *BF-1* coding sequence. This was followed by cloning a *PvuII* fragment into the blunt *Bam*HI site of pGEX-3X. For expression of GST fusion proteins in mammalian cells, plasmid pEBG-Hes1(3–281) was obtained as described previously (39), while construct pEBG-Hes1(Δ 276–281) (truncated Hes1 lacking the last six amino acids, WRPWRN) was obtained by subcloning the appropriate PCR product into the filled-in *Bam*HI site of pEBG. pEBG-TLE1(1–770) (full-length TLE1) was generated by subcloning the entire TLE1 coding region into the filled-in *Bam*HI site of pEBG. pEBG-Engrailed1 (full-length Engrailed1) was generated by subcloning a filled-in *Bam*HI fragment into the filled-in *Bam*HI site of pEBG. For in vitro translation reactions, plasmid p β glob-BF-1(1–481) (full-length BF-1) was generated by digestion with *PvuII* and *XhoI*, followed by ligation to the *pT7 β glob* vector, containing the β -globin gene 5' untranslated sequence. Plasmid p β glob-BF-1(1–336) was obtained by digesting p β glob-BF-1(1–481) with *HincII*, while

construct p β glob-BF-1(120–481) was generated using BAL 31 nuclease; subsequent restriction digestion of this plasmid with *SfiI* generated construct p β glob-BF-1(120–275). Plasmid pcDNA3-GAL4bd-BF-1(241–336) (fusion protein of the DNA-binding domain of GAL4 [GAL4bd] and amino acids 241 to 336 of BF-1) was obtained by digesting the *BF-1* cDNA with *XmaI* (followed by filling in with T4 DNA polymerase) and *HincII* and subcloning into the *EcoRV* site of pcDNA3-GAL4bd (18, 19). The expression vector pCMV2-FLAG-BF-1(1–481) was generated by subcloning a *PvuII/XhoI* fragment of the *BF-1* cDNA into the *EagI* and *SalI* sites of pCMV2-FLAG. Constructs pCMV2-FLAG-Hes1(1–281), pCMV2-FLAG-Hes1(Δ 276–281) (39), pcDNA3-TLE1(1–770) (19), p6B-CMV-Luc (luciferase gene under the control of the cytomegalovirus [CMV] promoter linked to six BF-1 binding sites) (35) and p6N- β Act-Luc (luciferase gene under the control of the β -actin promoter linked to six Hes1 binding sites) (44) have been described previously. The histone deacetylase 1 (HDAC1) expression plasmid pT7-HA-HDAC1 was kindly provided by X. J. Yang (McGill University).

Interaction assays in transfected cells and Western blotting analysis. Human 293 or rat ROS17/2.8 cells were grown and transfected using the SuperFect reagent (Qiagen) as described previously (39). Coprecipitation assays using plasmids pEBG-TLE1(1–770), pEBG-Hes1(3–281), pEBG-Hes1(Δ 276–281), pEBG-Engrailed1 (or pEBG as control), and pCMV2-FLAG-BF-1(1–481) and immunoprecipitation experiments with anti-FLAG (Sigma) or anti-hemagglutinin (HA) (Boehringer) epitope antibodies were performed as described previously (25, 39). Polyclonal antibodies against BF-1 were obtained by immunizing rabbits with the peptide CTHQNQGSSSNPLIH, containing the last 14 amino acids of BF-1 and an amino-terminal Cys residue to permit linking to keyhole limpet hemocyanin. Antibodies were purified from serum by 40% ammonium sulfate precipitation, followed by affinity purification on a BF-1 peptide-Sepharose column. Western blotting studies were performed as described elsewhere (18, 25, 39, 42) with antibodies against BF-1, TLE1 (25, 54, 55), GST (Santa Cruz Biotechnology), FLAG epitope, or HDAC1 (Santa Cruz Biotechnology) or with pan-TLE monoclonal antibodies (42, 47). Rabbit polyclonal antibodies against Hes1 were kindly provided by J. Feder (Bristol-Myers Squibb) and used for Western blotting as described previously (6, 8).

Coimmunoprecipitation of BF-1 and TLE from mouse embryonic telencephalon. The telencephalon from embryonic day 15 mouse embryos was dissected as described elsewhere (17, 46). Tissue was rinsed in ice-cold Hanks' balanced salt solution, followed by homogenization and whole-cell lysis (25). Lysates were subjected to immunoprecipitation with either anti-TLE1 serum or preimmune serum as described previously (25), and immunoprecipitated material was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting with anti-BF-1 and pan-TLE antibodies.

Primary cultures of telencephalic neural progenitor cells. Neural progenitor cell cultures were obtained from telencephalic cortices dissected from embryonic day 12.5 mouse embryos exactly as described previously (17, 46). Cell lysates were prepared after 24 h in vitro when ~90% of the cultured cells were mitotic (as indicated by bromodeoxyuridine incorporation studies [reference 46 and data not shown]).

In vitro fusion protein interaction assays. Incubations of in vitro-translated proteins and bacterially purified GST fusion proteins were performed as described elsewhere (18, 39).

Transcription assays. ROS17/2.8 or 293 cells were transfected using the SuperFect reagent as described previously (39). The amount of DNA transfected was adjusted using plasmid pcDNA3 so that the total amount of DNA used in each transfection was the same (3.0 μ g). Transcription studies were performed with 500 ng of reporter plasmid p6B-CMV-Luc or p6N- β Act-Luc. Effector plasmids included pCMV2-FLAG-BF-1(1–481) (10 ng), pcDNA3-TLE1(1–770) (400 ng), pCMV2-FLAG-Hes1(1–281) (25 ng), and pCMV2-FLAG-Hes1(Δ 276–281) (25 ng). In each case, 250 ng of pCMV- β -galactosidase plasmid DNA was cotransfected to provide a means of normalizing the assays for transfection efficiency. Results were expressed as mean value $\pm$ standard deviation (SD) and were tested for statistical significance by the one-tailed Student's *t* test for paired differences.

EMSA. The oligonucleotide probes used in electrophoretic mobility shift assays (EMSAs) contained either two Hes1 binding sites (N box [44]) (top strand, 5'-CTAGACGCCACGAGCCACAGGATTG-3'; bottom strand, 5'-CTAGCAATCCTTGTGGCTCGTGGCGT-3') or one BF-1 binding site (B2 probe [(35)]) (top strand, 5'-TCGAGCTCAATGTGAACAGAGCAG-3'; bottom strand, 5'-CTGCCTGCTCTGTTTACATTGGAGC-3') (binding sites are italicized). Probes were labeled at both ends by filling in with Klenow DNA polymerase in the presence of [α -³²P]dCTP. EMSA-s were performed as described elsewhere (45), using BF-1 prepared by in vitro translation.

FIG. 1. Interaction of BF-1 and TLE proteins in vivo. Mouse embryonic telencephalon was dissected and homogenized; a whole-cell lysate prepared and used for immunoprecipitation (IP) with either anti-TLE1 rabbit serum (lane 2) or preimmune rabbit serum (lane 3). Immunoprecipitated material was subjected to SDS-PAGE on a 7% gel together with 1/10 of the input lysate (lane 1), followed by Western blotting (WB) with either rat pan-TLE monoclonal antibodies (A) or rabbit anti-BF-1 polyclonal antibodies (B). (A) The secondary antibodies used to detect the rat pan-TLE antibodies cross-react weakly with the rabbit immunoglobulin G heavy chain (IgG HC). Here and in succeeding figures, positions of size standards are indicated in kilodaltons

RESULTS

In vivo interaction between BF-1 and TLE proteins. Previous studies have implicated vertebrate BF-1 family members in transcriptional repression (5, 35). The biological effects of full-length BF-1 can be phenocopied with fusion proteins of the BF-1 DNA-binding domain and the Engrailed transcription repression domain (5). The Engrailed repressor domain mediates interactions with Groucho/TLE proteins (29, 50), suggesting that BF-1 may act as a transcriptional repressor together with Groucho/TLEs. This possibility is consistent with the previous demonstration that mouse *BF-1* is coexpressed with *TLE* genes in neural progenitor cells of the telencephalon (11, 48, 53–55). To begin to determine whether TLE proteins might act as corepressors with BF-1, we examined whether these molecules could interact in vivo. Mouse embryos were collected 15 days postcoitum; the telencephalon was dissected, followed by homogenization, preparation of a whole-cell lysate, and immunoprecipitation with previously described (25, 54, 55) anti-TLE1 antibodies. Western blotting analysis showed that TLE proteins were immunoprecipitated by these antibodies (Fig. 1A, lane 2) but not by preimmune serum (Fig. 1A, lane 3). More importantly, BF-1 was coimmunoprecipitated by the anti-TLE1 antibodies (Fig. 1B, lane 2) but not by preimmune serum (Fig. 1B, lane 3). These findings show that BF-1 interacts with TLE proteins in vivo.

An interaction between BF-1 and TLE was also observed when protein-protein interaction assays were performed with transfected human embryonic kidney 293 cells. Cells were cotransfected with expression plasmids for mouse BF-1 and a fusion protein of GST and full-length TLE1 (a plasmid expressing GST alone was used as control). Twenty-four hours later, cells were homogenized and the GST proteins were iso-

lated on glutathione-Sepharose beads. Western blotting analysis of the fractions bound to the beads using anti-BF-1 antibodies showed that BF-1 coprecipitated with GST-TLE1 (Fig. 2A, lane 2) but not with GST (Fig. 2A, lane 4), indicative of an interaction between BF-1 and TLE1. Both GST and GST-TLE1 were stable and expressed at equivalent levels (Fig. 2B). Coimmunoprecipitation assays were performed next. Cells were transfected with GST-TLE1, with or without a FLAG epitope-tagged BF-1 protein (FLAG-BF-1). Cell homogenates were subjected to immunoprecipitation with anti-FLAG antibodies, and the immunoprecipitated proteins were tested for the presence of TLE1 immunoreactivity by Western blotting with anti-TLE1 antibodies. GST-TLE1 was immunoprecipitated when cells were cotransfected with FLAG-BF-1 (Fig. 2C, lane 1) but not when they were cotransfected with the empty FLAG expression plasmid (Fig. 2C, lane 2) or when immunoprecipitations were performed with irrelevant monoclonal antibodies (not shown). Endogenous TLE1 as well as previously described proteolytic fragments thereof (25) (Fig. 2C, lane 1) were also immunoprecipitated. Reprobing with anti-FLAG antibodies confirmed that FLAG-BF-1 coimmunoprecipitated with TLE1 (Fig. 2D, lane 1). Together, these results demonstrate that BF-1 interacts with TLE proteins.

Next, we asked whether BF-1 and TLE proteins could interact directly and, if so, which respective domains mediate their association. In vitro protein-protein interaction assays were performed using fusion proteins of GST and individual TLE domains purified from bacteria (Fig. 3) and full-length BF-1 [BF-1(1–481)] prepared by in vitro translation. BF-1(1–481) bound to the carboxy-terminal WDR domain of TLE (Fig. 4A, lane 4). A weaker interaction with the amino-terminal Q domain of TLE1 was also observed (Fig. 4A, lane 2). A truncated form of BF-1 lacking the last 145 amino acids [BF-1(1–336)] retained the ability to interact with these two domains (Fig. 4B, lanes 2 and 4). Similarly, deletion of the first 119 amino acids did not abolish binding of BF-1 to TLE (Fig. 4C). Longer exposures revealed that removal of this amino-terminal region was correlated with a weak interaction of BF-1(120–481) with the SP domain of TLE1 (partly visible in Fig. 4C, lane 3), although the significance of this observation remains to be determined. Contrary to BF-1(120–481), a truncated BF-1 form containing a further carboxy-terminal deletion, BF-1(120–275), was unable to interact with either TLE domain (Fig. 4D). These combined observations suggest that amino acids 276 to 336 of BF-1 are involved in TLE binding. In agreement with this possibility, a fusion protein of BF-1(241–336) and GAL4bd was competent to interact with the WDR domain of TLE (Fig. 4E, lane 4). A much weaker interaction with the Q domain was also observed (Fig. 4E, lane 2). GAL4bd does not interact with any of these TLE domains (18, 19). Taken together, these results demonstrate that BF-1 interacts with TLEs and that, barring the presence of a bridging protein in the rabbit reticulocyte lysate, this interaction is direct.

Functional interaction between BF-1 and TLE proteins. To determine whether BF-1 and TLE proteins could functionally interact, we asked whether the latter might be involved in the transcriptional functions of BF-1. Previous studies have shown that Groucho/TLEs are global transcriptional corepressors that can act as adapters between DNA-binding proteins and histone deacetylases (2, 9, 10, 14, 18, 34, 39). We therefore

FIG. 2. Interaction of BF-1 and TLE1 in mammalian cells. (A and B) Transfection-coprecipitation assays. Human 293 cells were cotransfected with plasmids encoding full-length BF-1 (lanes 1 to 4) and either a fusion protein of GST and full-length TLE1 (lanes 1 and 2) or GST alone (lanes 3 and 4). Cell homogenates were collected and incubated with glutathione-Sepharose beads. The material that remained bound to the beads after extensive washing was subjected to SDS-PAGE (lanes 2 and 4). One-tenth of each input homogenate collected prior to incubation with glutathione-Sepharose beads was also subjected to gel electrophoresis (lanes 1 and 3). After transfer to nitrocellulose, Western blotting (WB) was performed sequentially with either anti-BF-1 (A) or anti-GST (B) antibodies. (C and D) Coimmunoprecipitation studies. 293 cells were cotransfected with plasmids encoding the indicated combinations of proteins and peptides. Cell homogenates were subjected to immunoprecipitation (IP) with anti-FLAG epitope antibodies, and the material that was bound to the beads after extensive washing was subjected to SDS-PAGE, transfer to nitrocellulose, and Western blotting with anti-TLE1 antibodies (C) and then with anti-FLAG antibodies (D). (C) GST-TLE1 coimmunoprecipitates with FLAG-BF-1 (lane 1, closed arrow); endogenous TLE1 proteins (lane 1, arrowhead) and previously described proteolytic products thereof (25) (lane 1, open arrow) also coimmunoprecipitate with FLAG-BF-1. (D) After incubation with the anti-TLE1 antibodies, the nitrocellulose was not stripped before incubation with the anti-FLAG antibodies so that the same bands visible in panel C are still visible in panel D. The large arrow points to the position of migration of FLAG-BF-1 (lane 1). The immunoglobulin G heavy chain is indicated (IgG HC).

examined the effects of TLE proteins on BF-1-mediated repression by first testing whether overexpressing TLE1 would potentiate repression by BF-1. Rat ROS17/2.8 cells, which contain endogenous TLE proteins (39), were transfected with a previously described (35) reporter construct containing the luciferase gene under the control of a CMV promoter linked to six tandem copies of a BF-1 binding site. Transfection of this reporter plasmid (p6B-CMV-Luc) alone resulted in basal expression of the luciferase gene (Fig. 5, lane 1). Cotransfection of a BF-1 expression plasmid led to a repression of basal transcription (Fig. 5, lane 3). Importantly, cotransfection of a TLE1 expression plasmid resulted in a significant potentiation of the repressive effect of BF-1 (Fig. 5, cf. lanes 3 and 5). Neither BF-1 nor TLE1 repressed basal transcription from the CMV promoter alone (Fig. 5, lanes 7–9). These results are consistent with the direct interaction between TLE and BF-1 proteins demonstrated above and suggest that TLE proteins may promote BF-1-mediated transcriptional repression.

Next, we asked whether transcriptional repression by BF-1 was reduced in the presence of the histone deacetylase inhibitor trichostatin A (TSA) (49). TSA treatment had no significant effect on the activity of the CMV promoter (Fig. 5, cf.

lanes 1 and 2) but reduced the repressive activity of BF-1 (Fig. 5, cf. lanes 3 and 4), suggesting that histone deacetylases are involved in repression by BF-1. The inhibitory effect of TSA on BF-1 was partly alleviated by overexpression of TLE1 (Fig. 5, cf. lanes 4 and 6). This observation is in agreement with the suggestion that Groucho/TLEs may be able to mediate transcriptional repression also independently of histone deacetylases (9).

Based on the effect of TSA on repression by BF-1, we tested further whether BF-1 might associate with histone deacetylases in cultured cells. We focused on HDAC1, which has been shown previously to interact with Groucho/TLE proteins (10). 293 cells were transfected with BF-1, with or without a HA epitope-tagged HDAC1 protein (Fig. 6A). Immunoprecipitation experiments with anti-HA antibodies showed that both BF-1 (Fig. 6B, lane 1) and endogenous TLEs (Fig. 6C, lane 1) were coimmunoprecipitated with HDAC1 (Fig. 6D, lane 1), indicating that these proteins can interact with each other in vivo. To determine whether BF-1 might interact with HDAC1 directly, in vitro-translated HDAC1 was incubated with bacterially purified GST-BF-1 proteins. Isolation of GST-BF-1 did not result in a coisolation of HDAC1 (Fig. 6E). We also failed

FIG. 3. Expression of individual TLE domains in bacterial cells. (A) Schematic representation of the domain structure of TLE proteins and truncated derivatives thereof (47). The amino terminus of all Groucho/TLE proteins contains a Q domain that mediates protein-protein interactions and transcriptional repression, followed by a GP domain, an internal region involved in nuclear localization (CcN domain), and an SP domain involved in transcriptional repression. The carboxy-terminal half of Groucho/TLE proteins is highly conserved and contains a WDR domain that mediates protein-protein interactions. Also shown are deletion derivatives of TLE1 and TLE3 used in this study, named according to the residues contained in each protein. (B) Analysis of individual GST-TLE fusion proteins by SDS-PAGE. Roughly equivalent amounts of GST-TLE1(1-135) (lane 2), GST-TLE1(290-461) (lane 3), GST-TLE3(490-774) (lane 4), or GST (lane 5) were visualized by staining with Coomassie blue.

to detect an interaction between BF-1 and HDAC1 in far-Western blotting studies (data not shown). Taken together, these results suggest that BF-1 can recruit complexes containing TLE and HDAC1 proteins to repress transcription.

Association of BF-1 and Hes1. Our present and previous (18, 40) findings show that TLE proteins can interact with both BF-1 and Hes1. Moreover, TLE, BF-1, and Hes1 are coexpressed in telencephalic neural progenitor cell cultures (Fig. 7). We therefore asked whether, due to their shared ability to interact with TLEs, BF-1 and Hes1 might form a complex. Since we were unable to immunoprecipitate Hes1 from telencephalic neural progenitor cells with either anti-TLE or anti-Hes1 antibodies (data not shown), protein-protein interaction assays were performed with transfected cells. ROS17/2.8 cells were cotransfected with BF-1 and either GST, a fusion protein of GST and Hes1 [GST-Hes1(3-281)], or a fusion protein of GST and a truncated form of Hes1 [GST-Hes1(Δ 276-281)] that lacked the last six amino acids, WRPWRN, necessary for TLE binding (14, 40) (Fig. 8A). After affinity isolation of the GST proteins, Western blotting analysis showed that BF-1 coisolated with GST-Hes1(3-281) (Fig. 8B, lane 2) but not with GST-Hes1(Δ 276-281) (Fig. 8B, lane 4) or GST (Fig. 8B,

lane 6). Similarly, endogenous TLE proteins coprecipitated with Hes1(3-281) (Fig. 8C, lane 2) but not with Hes1(Δ 276-281) (Fig. 8C, lane 4). BF-1 and Hes1 did not interact directly with each other in *in vitro* protein-protein interaction assays in which GST-Hes1 was isolated from bacteria (which do not express TLEs) and BF-1 was prepared by *in vitro* translation using a reticulocyte system devoid of TLEs (39) (Fig. 8D). Under the same conditions, GST-Hes1 interacted with *in vitro*-translated TLE proteins (Fig. 8E). In agreement with previous studies (18), the TLE-Hes1 interaction was relatively weak *in vitro* compared to similar assays in transfected cells (cf. Fig. 8C and E). These results suggest that BF-1 and Hes1 can associate in mammalian cells and that their interaction is not direct but mediated by TLE proteins.

To determine the specificity of the BF-1-Hes1 interaction, we tested whether other proteins known to interact with TLE family members might also associate with BF-1. Similar protein-protein interaction studies were performed by cotransfecting cells with BF-1 and either a fusion protein of GST and Engrailed1 or GST alone (Fig. 9C). Endogenous TLE proteins were coprecipitated with GST-Engrailed1 (Fig. 9A, lane 3) but not with GST (Fig. 9A, lane 4). In contrast, BF-1 did not associate with GST-Engrailed1 (Fig. 9B, lane 3). The lack of an association between Engrailed and BF-1 suggests further that the BF-1-Hes1 interaction is specific.

Potential of Hes1-mediated transcriptional repression by BF-1. To examine the interaction of BF-1 and Hes1 further, we next tested whether BF-1 could modulate the transcription repression function of Hes1. 293 cells were transfected with the previously described (44) p6N- β Act-Luc reporter construct, containing the luciferase gene under the control of a basally active β -actin promoter linked to six tandem copies of a Hes1 binding site. Cotransfection of limited amounts of full-length Hes1 resulted in a partial repression of basal transcription (Fig. 10A, cf. lanes 1 and 2). Importantly, the repressor activity of Hes1 was significantly enhanced by BF-1 (Fig. 10A, lane 3), while BF-1 had no effect on reporter gene expression in the absence of Hes1 (Fig. 10A, lane 6). In contrast, equal amounts of Hes1(Δ 276-281) did not mediate transcriptional repression (Fig. 10A, lane 4), in agreement with the notion that removal of the TLE-binding domain impairs the ability of Hes proteins to repress transcription (40). The presence of BF-1 did not promote repression by Hes1(Δ 276-281) (Fig. 10A, lane 5), consistent with the finding that BF-1 and Hes1(Δ 276-281) do not associate with each other in transfected cells. These results show that BF-1 can potentiate the ability of Hes1 to mediate transcriptional repression and that this effect is dependent on the presence of the TLE-binding domain of Hes1. The six tandem N boxes present in the p6N- β Act-Luc reporter construct do not contain any sequence resembling the consensus BF-1 binding site (35), suggesting that the potentiating effect of BF-1 on Hes1 was not due to a direct binding of BF-1 to the reporter construct. To confirm this possibility, EMSAs were performed using previously described oligonucleotide probes containing either one copy of the BF-1 binding site (BF-1 probe [35]) or two tandem copies of the N-box (Hes1 probe [44]). As previously described (35), the electrophoretic mobility of the BF-1 probe was retarded in the presence of BF-1 (Fig. 10B, lane 2); this retardation was not observed when an excess of unlabeled probe was present (Fig. 10B, lane 3). In

FIG. 4. Interaction of BF-1 with separate TLE domains. Shown on the left are schematic representations of the in vitro-translated ^{35}S -labeled BF-1 proteins used in these assays. The hatched box indicates the location of the winged-helix domain (WHD). Lane 1 of each corresponding SDS-PAGE gel shown on the right was loaded with 40% of the amount of in vitro-translated protein used in the binding assays (Input). Lanes 2 to 5 show the products of the pull-down experiments performed in the presence of $\sim 1.0 \mu\text{g}$ of the indicated fusion proteins. (E) Amino acids 241 to 336 of BF-1 were expressed as a fusion protein with GAL4bd. The material that was recovered on glutathione-Sepharose beads was subjected to SDS-PAGE and autoradiography. Gels were loaded leaving empty lanes between individual samples, except between lanes 2 and 3 in panel A.

contrast, no DNA-protein complexes were observed when the Hes1 probe was incubated in the presence of BF-1 (Fig. 10B, lane 4). Together, these results are consistent with a model in which BF-1 can associate with Hes1 through TLE proteins and participate in Hes1-mediated repression in the absence of its own DNA-binding sites.

DISCUSSION

Functional interaction between BF-1 and Groucho/TLE proteins. In an effort to elucidate the molecular mechanisms that underlie the functions of BF-1, we have examined the possibility that this factor is involved in the regulation of gene expression together with Groucho/TLE family members. Our studies have provided the first demonstration that BF-1 and TLE proteins can physically interact with each other in vivo. Their interaction appears to be direct, since it was also observed in binding assays using bacterially purified TLE proteins

and in vitro-translated BF-1 preparations. Two separate TLE domains, the amino-terminal Q domain and the carboxy-terminal WDR region, are involved in BF-1 binding. This finding is in agreement with previous investigations showing that these two domains mediate protein-protein interactions with a number of other factors, including RUNX (39), NK-3 (10), and UTY (19) proteins. These observations suggest that the use of multiple protein-protein interaction domains is a strategy regularly utilized by Groucho/TLEs, perhaps to achieve a specificity that may not be provided by each interaction domain alone. Our studies have shown further that a short region of BF-1, located immediately after the winged-helix domain, is involved in TLE binding. Interestingly, we have noticed that this region contains a sequence, YWPMSPFLSLH, that is conserved among all BF-1 family members (5) and is characterized by two adjacent aromatic residues followed by the motif PFLSL (underlined). This arrangement of aromatic residues separated by one or two proline residues is reminiscent of the

FIG. 5. Effect of TLE and histone deacetylase activities on transcriptional repression by BF-1. ROS17/2.8 cells were transfected with the reporter construct p6B-CMV-Luc (500 ng; lanes 1 to 6) and the indicated expression vectors in the absence (lanes 1, 3, and 5) or presence (lanes 2, 4, and 6) of TSA (400 nM). Expression vectors used were pCMV2-FLAG-BF-1 (10 ng per transfection; lanes 3 to 6) and pcDNA3-TLE1 (400 ng; lanes 5 and 6). The basal activity of the reporter construct in the absence of BF-1 was considered 100%. Luciferase activities were expressed as the mean $\pm$ SD of at least four independent experiments performed in duplicate. BF-1 mediates transcriptional repression (lane 3; *, $P = 0.00011$), and this effect is enhanced by TLE1 (lane 5; *, $P = 0.00923$) and relieved by TSA (lanes 4 and 6). Neither BF-1 (lane 8) nor TLE1 (lane 9) had a repressive effect on a control reporter (pCMV-Luc; 500 ng) containing the CMV promoter but no BF-1 binding sites.

bona fide Groucho/TLE-binding motif WRP(W/Y) found in Hes and RUNX family members. Importantly, a similar sequence, YAFNHPFSINN, is present in the CRII region that mediates the interaction of TLEs with the winged-helix protein hepatic nuclear factor 3 β (51). Thus, it is possible that these short sequences may perform the common task of mediating the interaction of these winged-helix proteins with TLEs.

The present investigations have also demonstrated that the ability of BF-1 to mediate transcriptional repression is promoted by TLEs. This finding is in line with the previous demonstration that Groucho/TLEs provide a transcriptional corepressor function to other DNA-binding proteins (2, 10, 14, 18, 34, 40). It is also consistent with the demonstration that ectopic expression of XBF-1 in developing *Xenopus* embryos has effects that can be phenocopied by the ectopic expression of a fusion protein of BF-1 and the transcription repression domain of Engrailed (5). Since the Engrailed repression region was shown to act as a Groucho/TLE-binding domain (29, 50), those findings also suggest that BF-1 and TLE proteins repress transcription together. Additional support to this possibility derives from our present observation that BF-1 can potentiate transcription repression mechanisms that require TLE function. More specifically, BF-1 enhances repression mediated by Hes1 but has no effect on a truncated form of Hes1 that lacks the ability to interact with TLEs (see below for further discussion). Taken together, these results are consistent with the notion that BF-1 and TLE proteins form transcription repression complexes together.

BF-1 also associates with HDAC1 in mammalian cells. This interaction is not direct and may be mediated by TLE proteins, which can bind to both BF-1 and HDAC1. Importantly, BF-1-mediated transcriptional repression is reduced by an inhibitor

FIG. 6. Interaction of BF-1 and HDAC1 in transfected cells. (A to D) 293 cells were cotransfected with plasmids encoding full-length BF-1 (lanes 1 and 2) and either HA epitope-tagged HDAC1 (lane 1) or empty pCMV2-HA vector (lane 2). (A) Cell homogenates were subjected to Western blotting (WB) analysis with anti-BF-1 antibodies. (B to D) Cell homogenates were subjected to immunoprecipitation (IP) with anti-HA epitope antibodies, and the material that was bound to the beads after extensive washing was subjected to SDS-PAGE, transfer to nitrocellulose, and Western blotting (WB) with anti-BF-1 antibodies (B), pan-TLE antibodies (C), or anti-HDAC1 antibodies (D). (E) In vitro pull-down assays. In vitro-translated 35 S-labeled HDAC1 (lane 1; 15% of the amount used in each reaction) was incubated in the presence of ~ 2.0 μ g of either GST alone (lane 2) or GST-BF-1 (lane 3). The material that was recovered on glutathione-Sepharose beads was subjected to SDS-PAGE and autoradiography. No specific binding of HDAC1 to GST-BF-1 was observed, even after prolonged autoradiography.

of histone deacetylase activities. Thus, we propose that BF-1 can recruit TLEs and histone deacetylases to repress transcription, a possibility consistent with previous studies showing that histone deacetylases are involved in transcriptional repression mediated by Groucho/TLE proteins (9, 10). It remains to be determined, however, whether the recruitment of histone deacetylase activity represents the general mechanism normally utilized by BF-1 to repress transcription or whether other mechanisms may also be utilized. For instance, it will be important to determine whether Groucho/TLE proteins are always involved in repression by BF-1 or whether the latter can also repress transcription independently of the former. Moreover, Groucho/TLEs may contribute to BF-1 mediated repres-

FIG. 7. Expression of BF-1, TLE, and Hes1 in telencephalic neural progenitor cells. The telencephalon was dissected from embryonic day 12.5 mouse embryos, and primary cultures of cortical progenitor cells were established as described elsewhere (46). After 24 h in vitro, whole-cell lysates were prepared and subjected to Western blotting analysis with antibodies against TLE (A), BF-1 (B), or Hes1 (C).

sion in ways that may not always involve the recruitment of histone deacetylases.

Interaction of BF-1 and Hes1 proteins through TLE corepressors. Our studies have also provided the first evidence of an interaction between BF-1 and Hes1. These proteins can associate with each other in transfected cells, and their interaction is dependent on the ability of Hes1 to bind to TLEs, suggesting that the latter act as adapters between BF-1 and Hes1. This association appears to be specific because other proteins that bind to TLEs, like Engrailed, do not associate with BF-1 in the presence of TLEs. These findings suggest that in addition to acting as transcriptional corepressors, TLE proteins may contribute to the transcriptional functions of BF-1 by acting as adapters between BF-1 and Hes1. The modular structure of Groucho/TLEs, particularly the presence of tandem WD40 repeats capable of providing multiple protein-protein interaction surfaces, appears to be suited to this task. Our studies have shown further that BF-1 enhances the ability of Hes1 to repress transcription in transfected cells. This function appears to be mediated by TLEs, because BF-1 does not promote the transcription repression function of a carboxy-terminally truncated Hes1 protein that lacks the WRPW motif required for TLE binding. Interestingly, the potentiating effect of BF-1 on Hes1-mediated repression does not require binding of BF-1 to DNA, because it can occur even in the absence of BF-1 binding sites. Taken together, these observations suggest that complexes of BF-1, TLE, and Hes1 proteins may be involved in the regulation of overlapping sets of genes. This possibility is consistent with the results of separate lines of studies that have recently implicated both BF-1 and Hes1 in the regulation of the expression of cell cycle inhibitors of the Cip/Kip family (6, 21), although the involvement of the WRPW motif of Hes1 in these events remains to be determined.

Implications for the function of BF-1 during telencephalon development. Previous studies have shown that BF-1 is an important regulator of the progenitor-to-neuron transition in the mammalian telencephalon. In the absence of *BF-1*, telencephalic progenitor cells differentiate prematurely, leading to early depletion of the progenitor population (53). These findings suggest that BF-1 promotes cell proliferation and/or in-

FIG. 8. Interaction of BF-1 and Hes1 in mammalian cells. (A to C) Transfection-coprecipitation assays. ROS17/2.8 cells were cotransfected with plasmids encoding full-length BF-1 (lanes 1 to 6) and either a fusion protein of GST and full-length Hes1 [GST-Hes1(3-281); lanes 1 and 2], a fusion protein of GST and Hes1(Δ 276-281) (lanes 3 and 4), or GST alone (lanes 5 and 6). Cell homogenates were collected and incubated with glutathione-Sepharose beads. The material that remained bound to the beads after extensive washing was subjected to SDS-PAGE (lanes 2, 4, and 6). One-twentieth of each input homogenate collected prior to incubation with glutathione-Sepharose beads was also subjected to gel electrophoresis (lanes 1, 3, and 5). After transfer to nitrocellulose, Western blotting was performed with either anti-GST (A), anti-BF-1 (B), or pan-TLE monoclonal (C) antibodies. (D and E) In vitro pull-down assays. In vitro-translated 35 S-labeled BF-1 (lane 1 in panel D; 40% of the amount used in each reaction) or TLE1 (lane 1 in panel E; 20% of the amount used in each reaction) was incubated in the presence of ~ 1.0 μ g of either GST-Hes1(3-281) (lane 2) or GST alone (lane 3). The material that was recovered on glutathione-Sepharose beads was subjected to SDS-PAGE and autoradiography. No specific binding of BF-1 to GST-Hes1 was observed, even after prolonged autoradiography.

hibits cell differentiation in the telencephalon. BF-1 does not appear to have a direct growth-promoting activity, however, since disruption of *BF-1* function in *BF-1*^{-/-} mice has a demonstrable effect on the proliferation of neuroepithelial cells only after embryonic day 10.5, even though *BF-1* is expressed in these cells at earlier stages (53). It is possible that BF-1 may act as a regulator of the activities of growth-regulatory signals. Support to this hypothesis derives from the finding that the loss of *BF-1* leads to ectopic expression of *BMP4* in the telencephalic neuroepithelium (13, 53). This observation suggests that BF-1 may, at least in part, facilitate proliferation by inhibiting *BMP4* expression, since *BMP4* was shown to inhibit telence-

FIG. 9. Interaction of Engrailed1 with TLEs but not BF-1. (A and B) ROS17/2.8 cells were cotransfected with plasmids encoding full-length BF-1 (lanes 1 to 4) and either a fusion protein of GST and Engrailed1 (lanes 1 and 3) or GST alone (lanes 2 and 4). Cell homogenates were collected and incubated with glutathione-Sepharose beads. The material that remained bound to the beads after extensive washing was subjected to SDS-PAGE (lanes 3 and 4). One-twentieth of each input homogenate collected prior to incubation with glutathione-Sepharose beads was also subjected to gel electrophoresis (lanes 1 and 2). After transfer to nitrocellulose, Western blotting (WB) was performed with either pan-TLE monoclonal (A) or anti-BF-1 (B) antibodies. (C) GST-Engrailed1 (lane 1) and GST (lane 2) proteins isolated on glutathione-Sepharose beads.

phalic progenitor cell proliferation (16). In addition, recent studies in *Xenopus* have suggested that XBF-1 may be a direct regulator of the *p27^{Xic1}* gene, the amphibian counterpart of the mammalian cell cycle inhibitor *p27^{Kip1}* (21).

Based on our present demonstration that BF-1 forms transcription repression complexes with TLE and Hes1 proteins, we propose that BF-1 may control telencephalon development by coordinating the control of cell proliferation with the timing of differentiation in the neuroepithelium. In both invertebrates and vertebrates, Hes and Groucho/TLE proteins act as negative regulators of neuronal differentiation by preventing progenitor cells from differentiating prematurely (12, 23, 26–28, 41). The finding that BF-1 interacts with and enhances the

FIG. 10. Potentiation of Hes-1-mediated transcriptional repression by BF-1. (A) Transient transfection-transcription assays. 293 cells were transfected with reporter construct p6N-βAct-Luc (500 ng) and expression vectors encoding the indicated proteins. Expression vectors used were pFLAG-Hes1(1–281) (25 ng), pFLAG-Hes1(Δ276–281) (25 ng), and pCMV2-FLAG-BF-1 (10 ng). The basal activity of the reporter constructs in the absence of any cotransfected protein was considered 100%. Luciferase activities were expressed as the mean $\pm$ SD, of at least four independent experiments performed in duplicate. BF-1 enhances transcriptional repression by Hes1(1–281) (lane 3; *, $P = 0.0049$) but has no effect on Hes1(Δ276–281) (lane 5). (B) EMSAs. BF-1 was prepared by in vitro translation and incubated in the presence of either the radiolabeled BF-1 oligonucleotide probe (lane 2), the radiolabeled BF-1 probe plus a 200-fold molar excess of unlabeled BF-1 probe (lane 3), or the radiolabeled Hes1 probe (lane 4). Lane 1 was loaded with the BF-1 probe alone. The arrow points to the DNA-protein complex that was observed when BF-1 was incubated with the BF-1 probe alone but not in the presence of an excess of unlabeled probe.

transcription repression activity of Hes1 suggests that BF-1 may contribute to the regulation of the timing of neuronal differentiation together with Hes1 and TLE proteins. This possibility is consistent with the demonstration that *Hes1^{-/-}* mice display a forebrain phenotype very similar to that of *BF-1^{-/-}* mice, namely, premature differentiation of precursor cells with consequent depletion of the progenitor cell population (27). In the future, it will be important to determine whether BF-1 is involved in the regulation of the expression of genes that are thought to be targets of the transcriptional inhibitory functions of Hes proteins, like the proneural gene *Mash1* (8, 27).

A functional interaction between BF-1 and Hes1 may also help explain the results of studies of *Xenopus* embryos showing that ectopic expression of high doses of *XBF-1* causes suppression of neuronal differentiation in the injected area in a cell autonomous way (5). It is conceivable that the BF-1–Hes1 interaction may be favored in cells expressing high doses of BF-1. As a result, the inhibitory function of Hes1 during neuronal differentiation may be promoted due to the potentiation of its transcription repression activity, leading to suppression

of neuronal differentiation within the areas of high BF-1 expression. It remains to be determined, however, whether a similar situation may occur at lower *BF-1* concentrations. Studies in *Xenopus* showed that microinjection of low doses of *XBF-1* does not cause suppression of neuronal differentiation but instead leads to the formation of supernumerary neurons within the injected area (5). This observation suggests that at low concentrations, BF-1 may not be able to enhance Hes1 activity but may still be able to suppress the growth-inhibitory function of p27^{Kip1} and/or other antiproliferation factors. This would lead to increased proliferation without an antineurogenic effect, eventually resulting in supernumerary neurons when the progenitor cells differentiate. These observations suggest that changes in BF-1 protein levels may have important repercussions during the progenitor-to-neuron transition and underscore the importance of the mechanisms that regulate *BF-1* expression and function.

In summary, we propose that Groucho/TLE proteins are involved in BF-1 activity both by providing a corepressor function and by acting as adapters between BF-1 and Hes proteins. The establishment of transcription complexes containing BF-1 and Hes proteins may provide a way to integrate the functions of these factors, thereby coordinating the decision of precursor cells to exit the cell cycle and initiate the neuronal differentiation program.

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