Published in final edited form as:

J Neurosci Res. 2008 August 15; 86(11): 2423–2431. doi:10.1002/jnr.21680.

Histone deacetylase-related protein inhibits AES-mediated neuronal cell death by direct interaction

Xiaoguang Zhang, Hsin-Mei Chen, Eduardo Jaramillo, Lulu Wang, and Santosh R. D'Mello*
Department of Molecular and Cell Biology, University of Texas at Dallas, Richardson, TX 75080

Abstract

Histone deacetylase-related protein (HDRP), an alternatively-spliced and truncated form of HDAC9 that lacks a C-terminus catalytic domain, protects neurons from death. In an effort to understand the mechanism by which HDRP mediates its neuroprotective effect, we screened for proteins in the brain that interact with HDRP using a yeast two hybrid assay. One of the HDRP-interacting proteins identified in this screen was Amino Enhancer of Split (AES), a 197-amino acid protein belonging to the Groucho family. Interaction between HDRP and AES was verified by *in vitro* binding assays, co-immunoprecipitation and co-localization studies. To investigate the significance of the HDRP-AES association to the regulation of neuronal survival, we used cultured cerebellar granule neurons, which undergo apoptosis when treated with low potassium (LK) medium. We found that in contrast to HDRP whose expression is markedly reduced by LK treatment, AES expression was not appreciably altered. Forced expression of AES in healthy neurons results in cell death, an action that is blocked by the co-expression of HDRP. AES is a truncated version of larger Groucho-related proteins, one of which is TLE1. We found that the expression of TLE1 is reduced in LK-treated neurons and the forced expression of TLE1 blocks LK-induced neuronal death as well as death induced by AES. Our results suggest that AES has apoptotic activity in neurons and that neuroprotection by HDRP is mediated by the inhibition of this activity through direct interaction.

Keywords

apoptosis; neuronal survival; histone deacetylase; neurodegeneration; cerebellar granule neurons

INTRODUCTION

Apoptosis plays a critical role in development of the nervous system but can also cause unwanted neurodegeneration when aberrantly activated. Numerous molecules have been implicated in the regulation of neuronal apoptosis (D'Mello et al., 2005; Hara et al., 2006). Among these are histone deacetylases (HDACs), a family of proteins originally identified on the basis of their ability to deacetylate histones (de Ruijter et al., 2003; Verdin et al., 2003; Yang et al., 2005). Indeed, we recently reported that HDAC-related protein, HDRP, a spliced form of HDAC9 that lacks the catalytic domain, protects neurons from death (Morrison et al., 2006). Overexpression of HDRP blocks death of cerebellar granule neurons (CGNs) caused by low potassium (LK) treatment while forced suppression of HDRP expression induces death of neurons that are otherwise healthy. Furthermore, neurons cultured from HDRP-deficient mice are more vulnerable to apoptosis. We reported that forced expression of HDRP inhibits c-jun, a protein whose activation contributes to neuronal apoptosis (Morrison et al., 2006). As a step towards understanding the mechanisms underlying HDRP-mediated neuroprotection

*Corresponding author: Santosh R. D'Mello, Dept. of Molecular and Cell Biology, University of Texas at Dallas, 800 West Campbell, Richardson, TX 75080 Phone: (972) 883 2520. Fax: (972) 883 2409. Email: dmello@utdallas.edu.

more comprehensively, we initiated a search for proteins that interact with HDRP in the brain. In this report, we describe the identification of Amino Enhancer of Split (AES), as an HDRP-interacting protein.

AES is a 197-amino acid protein homologous to the *Drosophila* Groucho protein. Mammals express multiple homologs of Groucho referred to Transducin-like Enhancer of split (TLE) in humans (or Groucho-related genes, Grgs, in mice) which play crucial roles in the regulation of nervous system development as well as a number of developmental processes (Chen et al., 2000; Courey et al., 2001; Gasperowicz et al., 2005; Buscarlet et al., 2007). TLE proteins lack DNA-binding activity of their own but are recruited to specific gene regulatory sequences via interaction with a number of different DNA-binding transcription factors such as the HES class of basic HLH transcription factors, the LEF-1/TCF transcription factors which mediate Wnt signaling, Runt domain proteins, Dorsal, and Engrailed (Chen et al., 2000; Courey et al., 2001). TLE proteins can be divided into two subgroups. Members of one subgroup, TLE1–4, share common structural features including an amino terminal glutamine-rich region (Q domain), a glycine/proline-rich region (GP domain), a CcN domain containing phosphorylation sites for cdc2 and casein kinase II in close proximity of a nuclear localization sequence, a serine/proline-rich region (SP domain), and C-terminal WD40 repeats. Members of the other subgroup are truncated versions of these proteins lacking either the amino or carboxy terminus regions. AES (or Grg5 in mice) is the shortest member of this subgroup composed only of the Q domain and the GP domain. Mice lacking AES have been generated, and these mice exhibit a growth defect as well as skeletal abnormalities (Mallo et al., 1995; Wang et al., 2002). In this report we show for the first time that AES has pro-apoptotic activity and that HDRP thwarts the ability of AES to induce neuronal death by direct interaction. We also show that TLE1 protects neurons from death. Previous studies on the roles of TLE proteins in the nervous system have generally focused on early stages of brain development. Our study suggests that in mature neurons of the postnatal brain, these proteins function to regulate cell survival.

MATERIALS AND METHODS

Antibodies and other reagents

Cell culture media and chemicals were from Invitrogen and Sigma, respectively, unless specified otherwise. The following antibodies were used: Xpress (Invitrogen), His (Santa Cruz), Flag (Sigma), AES (Imgenex), c-Jun (Santa Cruz) and HDRP (Santa Cruz). All primary antibodies were used at 1:1000–1:5000. Secondary antibodies (Santa Cruz) were peroxidase-conjugated goat anti-rabbit IgG (1:10, 000) and goat anti-mouse IgG (1:10, 000).

Plasmids

The full-length HDRP and partial domain cDNAs corresponding to amino acids 1–454; 303–591; 178–591 were obtained by PCR from a human fetal brain library (Clontech). And fused in-frame to Gal4-DNA-binding domain using the pGBKT7 vector (Clontech) (Fig. 1B). Another set of HDRP GST-fusion expression plasmids were prepared using pGEX-KG vector (Clontech) with cDNAs corresponding to amino acids 1–343; 178–591 and 1–149 (Fig. 2B). Plasmid pcDNA3-Xpress/His-AES was obtained by PCR amplification of the region encoding amino acids 1 to 197 of human AES from human fetal brain cDNA library, and subcloning in frame into pcDNA3.1/His C (Invitrogen) (Fig. 2B). The PCR product of AES was also subcloned in-frame to GST using the pGEX-KG vector at the BamHI-Sall site (Fig. 2A). Primer sequences used for cloning available upon request. Flag-TLE1 mammalian expression vector was a gift from Dr. Michael G. Rosenfeld, Howard Hughes Medical Institute. Plasmid pTarget-Flag-HDRP was obtained by PCR amplification of Full length HDRP from mouse cDNA and subcloning to pTarget mammalian expression vector (Promega).

Cell culture

Human embryonic kidney cells (HEK293T) were purchased from ATCC (Manassas, VA) and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 50 U/mL penicillin and 50 µg/mL streptomycin. The Chinese Hamster Ovary cells (referred in the manuscript as CHO) were purchased from ATCC and were maintained in F-12K medium supplemented with 10% fetal bovine serum and 50 U/mL penicillin and 50 µg/mL streptomycin. CGNs were obtained from dissociated cerebella of 7–8 days old Wistar rats as described previously (D'Mello et al., 1993). Briefly, cells were plated in basal Eagle's medium (BME) supplemented with 10% fetal bovine serum, 25 mM KCl, 2 mM glutamine (Gibco-BRL) and 100 µg/mL gentamycin on dishes coated with poly L-lysine in 24-well dishes at a density of 1×10^6 cells/well, 1.2×10^7 cells/60 mm dish, or 3.0×10^7 cells/100 mm dish. After 18–22 h after plating, cytosine arabinofuranoside (10 µM) was added to the culture medium to prevent replication of non-neuronal cells. Cultures were maintained for 5–7 days prior to experimental treatments. Upon treatment, the cells were rinsed twice with serum-free BME medium and then maintained either in LK medium (serum-free BME medium, 5 mM KCl) or HK medium (serum-free BME medium, 25 mM KCl). Treatment of cultures with GW5074 (Sigma) was initiated at the time of switching the medium to LK as previously described (Chin et al., 2004). Cell viability was quantified by visualizing the morphology of the nuclei by staining chromatin with 4', 6'-diamidino-2-phenylindole hydrochloride (DAPI) as previously described (Morrison et al., 2006). Cells with condensed or fragmented nuclei were scored as apoptotic.

Yeast two-hybrid interaction screening

The yeast two-hybrid screening was performed according to the manufacturer's protocol (Clontech). The complete coding sequences of HDRP or partial domain were fused in-frame to the yeast Gal4 DNA-binding domain in the vector pGBKT7 (Clontech) to generate bait plasmids. Then the bait plasmids were tested for activation of the Gal4-dependent lacZ expression (β -galactosidase activity). Among them, full length HDRP and HDRP 1–154; 303–591; 178–591 bait plasmids displayed no intrinsic transcriptional activity, full length HDRP was chosen as bait for library screening. Yeast strain AH109 transformed with pGBKT7-HDRP was thus transformed with human fetal brain cDNA library, constructed with the yeast Gal4 activation domain vector pACT2 (Clontech) to screen for HDRP interacting proteins. Approximately 1.5×10^6 diploid yeast cells were selected on stringent condition plates with the dropout medium containing 5-bromo-4-chloro-3-indolyl- α -D-galactopyranoside (X- α -Gal) but lacking adenine, histidine, leucine, and tryptophan. 97 library plasmids were successfully recovered that were positive for β -galactosidase activity after cotransformation, and their identities established by sequencing. cDNA sequences were compared with GenBankTM and SwissProt databases for identification of the interacting proteins.

In Vitro Protein Binding Assay

Bacterially expressed GST-fusion proteins were prepared by transforming *Escherichia coli* strain, BL21 with either pGEXKG-AES, pGEXKG-HDRP1–343, pGEXKG-HDRP178–591 or pGEXKG-HDRP1–149 and grown overnight in LB medium with ampicillin (100 mg/ml). 0.5 ml of the culture was added to 300 ml of medium containing ampicillin and grown at 37 °C to reach A600 around 0.4–0.5 before adding 0.2uM isopropyl-1-thio-b-D-galactopyranoside (IPTG) to induce expression of fusion proteins. The culture was incubated at 37 °C for a further 3–5 h. The bacteria were harvested and lysed by sonication in 10 ml of NTEN (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, and 1mM dithiothreitol). And fusion proteins were purified by conjugating to glutathione-agarose beads (Sigma-Aldrich) according to the manufacturer's instructions. After extensive washing steps, immobilized fusion proteins on glutathione-agarose beads were directly used for in vitro binding assay without elution.

Binding assays were performed by using 5–10 µg of GST-fused protein immobilized on glutathione-agarose beads incubate with 5 to 10 µL of ³⁵S-labeled proteins in binding buffer (200 mM NaCl, 20 mM Tris-Cl, pH 7.6, 1mM EDTA) for 3h at 4 °C and washed three times with the same buffer. After removal of washing buffer, protein complexes were eluted by boiling with 20- 40µL of 2× SDS sample buffer (100 mM Tris-Cl, pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, and 200 mM beta-mercaptoethanol) and then analyzed by gel electrophoresis and autoradiography. *In vitro* synthesized HDRP and AES protein were synthesized with ³⁵S-methionine (MP Biomedicals) using TNT coupled lysate system (Promega).

Transfection and Immunoprecipitation

HEK293T cells were transfected with calcium phosphate method as described previously (Jan et al., 2004). CHO cells were transfected with Lipofectamine2000 according to the manufacturer's instructions (Invitrogen). The cells were harvested 48h after cotransfection and whole cell lysates were prepared in Triton X-100 lysis buffer [20 mM Tris pH 8.0, 140 mM NaCl, 1% Triton X-100 and 10% glycerol] containing protease inhibitors (Protease inhibitor cocktail tablets; Roche Diagnostics). The extract was incubated with 1 µg of primary antibody and 25 µl protein A–G beads (Santa Cruz) overnight at 4°C in Triton X-100 lysis buffer. The beads were pelleted and washed twice in Triton X-100 buffer and twice in wash buffer [20 mM Tris pH 8.0, 150 mM NaCl and 0.5% NP-40]. Then pellets were subjected to western blotting.

Western blotting

Protein concentrations were measured and normalized using Bradford protein assay reagent (Bio-Rad). 50–100 µg of cellular extracts or protein complex pulled down by GST fusion protein or antibodies were resolved on SDS-polyacrylamide gels. The proteins were then transferred to PVDF Membranes and then were blocked and incubated with the various primary antibodies mentioned previously. After washes, membranes were incubated with horseradish peroxidase-conjugated secondary antibody. Following the incubation, membranes were washed extensively and developed with ECL luminal reagent (Amersham Biosciences).

Reverse transcriptase PCR

RNA was isolated from cultured neurons using Trizol according to the manufacturer's instructions (Invitrogen). And PCR products were derived by using PCR master mix (Promega) with the following primers.

Rat TLE1 forward 5'-ATGTTCCCTCAGAGCCGG-3'

Rat TLE1 reverse 5'-AAGTGAGACTGCCCACTCAGA-3'

Rat AES forward 5'-GATGTTTCCGCAAAGCCG-3'

Rat AES reverse 5'-ATCGGACTTCTCTCCATCATC-3'

Rat β-actin forward, 5'-AGGACTCCTATGTGGGTGACGA-3'

Rat β-actin reverse, 5'-CGTTGCCAATAGTGATGACCTG-3'.

Rat HDRP forward, 5'-AACTTGAAGGTGCGGTCCA-3'

Rat HDRP reverse, 5'-TTACAAATCCTGGAGCTAAAT-3'

Neuronal transfections

Neuronal transfections were performed using the calcium phosphate method as described previously (Jan et al., 2004). Plasmid DNA was purified by cesium chloride–ethidium bromide gradients and was used at a concentration of 2 µg of DNA/ well for 24-well dishes and 3 µg DNA for 35 mm dishes of cultured CGNs. Transfections were generally performed on 4–5 days old cultures. For co-transfection experiments, 1 µg of each construct was used.

Immunocytochemistry

Cells were cultured on 24-well chamber slides (Nalgene Nunc Int.), fixed in PBS containing 4% paraformaldehyde for 15 min. After blocking in 0.1 M phosphate buffer containing 5% BSA and 5% goat serum for 60 min, immunostaining was performed by incubation with the primary antibody for 2h or overnight at 4°C, and the secondary antibody for 45 min at room temperature. The following primary antibodies were used: anti-FLAG (mouse, 1: 200), anti His (rabbit, 1: 100) and anti-Xpress (1: 100). The secondary antibodies for all experiments were Texas Red conjugated goat anti-mouse, FITC-conjugated goat anti-rabbit IgG. During washing of the secondary antibody, nuclei were stained in 1-µg/ml-4, 6-diamidino-2-phenylindole (DAPI) for 10 min at room temperature. Cover slips were mounted with fluormount (Molecular Probes). Double label immunohistochemistry was performed by incubating with Anti-Flag and Anti-His.

RESULTS

HDRP Interacts with the Groucho-related protein, AES

HDRP protects neurons from apoptosis (Morrison et al., 2006). Towards understanding the mechanisms involved, we proceeded to identify proteins in the brain that interacted with HDRP using the yeast two-hybrid approach. The entire human HDRP coding sequence was used as bait to screen a human embryonic brain library. Four of the 97 HDRP-interacting clones identified in the screening were found to encode AES. While two of these clones contained the complete coding sequence, the other two lacked 17 amino acids of the AES protein (Fig. 1A). The other HDRP-interacting clones identified in the screening will be described elsewhere. As a negative control in the screening process, we used the Class I HDAC protein, HDAC1, as bait. As shown in Fig. 1B, no interaction between AES and HDAC1 was detectable. To eliminate the possibility that the observed HDRP-AES interaction resulted in some way from the particular juxtaposition of the Gal4 activation domain with the cDNA sequence, a domain swap experiment was performed. HDRP was fused in-frame to the GAL4 activation domain and tested for its ability to interact with AES fused to the Gal4 DNA-binding domain. Transactivation of the reporter was retained (data not shown).

To determine the region within HDRP necessary for binding to AES, we generated deletion mutants fused to the Gal4 DNA-binding domain and introduced them into yeast along with pACT2-AES (encoding the completed coding sequence of AES) fused to the Gal4 transcriptional activation domain. While pGBKT7-HDRP clones containing regions of HDRP from amino acids 1–454 and 178–591 were positive for interaction, a clone containing the regions HDRP spanning amino acids 303–591 was not (Fig. 1B). These results indicate that the region between amino-acid 178 and 303 of HDRP is involved in interaction with AES.

In order to confirm the findings made using the yeast system, we performed an *in vitro* protein binding assay. ³⁵S-labeled *in vitro* translated full-length HDRP and truncated portions of the protein was synthesized using rabbit reticulocyte extract and tested for their ability to bind to bacterially expressed GST-AES fusion protein immobilized on glutathione-agarose beads. Full-length HDRP interacts with GST-AES, but not with GST alone (Fig. 2A). As shown in Fig. 2B, ³⁵S-labeled *in vitro* translated AES interacts with GST-HDRP 1–343 aa and 178–591

aa, but not with GST alone and GST-HDRP 1–149 aa. These results indicate that the region between amino-acids 178 and 343 of HDRP is involved in interaction with AES, which agree with the result from yeast cotransformation.

The physical interaction of HDRP and AES *in vitro* has little biological significance unless the two proteins localize to the same compartments of cells *in vivo*. HDRP is known to localize predominantly to the nucleus (Morrison et al., 2006). To examine the subcellular localization of AES in mammalian cells, CHO cells were transfected with a plasmid expressing Xpress/His-tagged AES. Visualization by immunofluorescence staining showed that AES is also expressed primarily within the nucleus (Fig. 3A).

To confirm the interaction between HDRP and AES in intact cells, we cotransfected CHO cells with the plasmid encoding Xpress/His-tagged AES along with one encoding Flag-tagged HDRP. Extensive overlap was observed in the staining pattern consistent with colocalization of the two proteins within the nucleus (Fig. 3A). To verify that HDRP interacts with AES in these cells, we performed a co-immunoprecipitation experiment. As shown in Fig. 3B, AES and HDRP coprecipitate confirming their interaction *in vivo*. The specificity of the antibodies utilized for coimmunoprecipitation was verified in control experiments (Supplementary data Fig 1).

AES has a pro-apoptotic effect in neurons

CGNs undergo apoptosis when switched from a medium containing depolarizing levels of potassium (referred to as HK medium) to medium containing low potassium (LK) (D'Mello et al., 1993). Although cell death begins only at about 16 hours, we and other investigators have determined that commitment to death occurs within 3 – 6 hours after LK treatment (Borodez et al., 1998; Galli et al., 1995; Nardi et al., 1997; Schulz et al., 1996). We have previously shown that the expression of HDRP is down regulated in neurons primed to die by LK treatment (Morrison et al., 2006). Having demonstrated that HDRP and AES proteins interact *in vitro* and in intact cells, we investigated whether the expression of AES was also down regulated during apoptosis. RT-PCR analysis revealed that AES mRNA expression was not substantially altered by LK treatment within the first 6 hours (Fig. 4A). The expression of c-jun, a well-established marker of neuronal death, is induced by LK indicating that the cultures are primed to die. The RT-PCR results were confirmed using Western blots (Fig. 4B).

Since HDRP and AES interact, a downregulation of HDRP would result in a relative excess of AES that is unbound by HDRP. To investigate the significance of such an imbalance to neuronal viability, we transfected neurons maintained in HK medium with an expression vector encoding AES. Overexpression of AES induced apoptosis in these normally healthy cultures as judged by condensed and/or fragmented nuclei (Fig. 5). The apoptotic effect of AES was confirmed by TUNEL-staining, an independent assay of cell viability (Supplementary data Fig 2A and C). LK-mediated neuronal death can be blocked by GW5074, a small molecule chemical inhibitor of c-Raf (Chin et al., 2004). Previous studies have shown that the mechanism by which GW5074 acts is different from that activated by HK. As shown in Fig. 5, GW5074-mediated neuronal survival is also neutralized by AES. These results confirm that AES has a pro-apoptotic activity in neurons (Fig. 5).

HDRP inhibits AES-induced neuronal death

The level of HDRP expression is relatively high in healthy cultures maintained in HK medium (Fig 4A, B; and Morrison et al., 2006). The pro-apoptotic action of AES along with the fact that HDRP expression is sharply reduced in neurons primed to die suggested that under normal circumstances HDRP maintains neuronal survival by inhibiting the pro-apoptotic activity of AES via direct interaction. If true, forced expression of HDRP could be expected to neutralize

the ability of AES to promote apoptosis. To test this model we co-transfected neurons with AES and HDRP. As predicted, the effectiveness of AES to promote apoptosis is substantially reduced when HDRP is co-expressed (Fig.6). Consistent with the model that inhibition of the pro-apoptotic action of AES by HDRP involves its direct interaction with AES is that the two proteins colocalize within the nucleus of LK-treated neuronal cultures (Fig.6A).

TLE1 inhibits AES-induced neuronal death

AES is a truncated version of a subgroup of larger Groucho proteins, TLE1–4 (referred to as Grg 1–4 in mice). We examined the expression of these TLE proteins in neurons primed to die by LK-treatment. As shown in Fig. 4A, the expression of TLE1 is downregulated in LK-treated neurons. Previous studies performed in other biological systems have revealed that AES and TLE1 have opposing affects and that AES antagonizes the action of TLE1 by acting in a dominant-negative fashion (Jan et al., 2004; Roose et al., 1998; Ren et al., 1999). If a similar relationship was operational in neurons, it could be predicted that the overexpression of TLE1 would have an anti-apoptotic effect and that TLE1 would be capable of interfering with the ability of AES to promote neuronal death. This was found to be the case. As shown in Fig.7, the overexpression of TLE1 in CGNs blocks LK-mediated neuronal death. TUNEL-staining confirmed the anti-apoptotic action of TLE1 (Supplementary data; Fig. 2B and C). Moreover, when co-expressed along with AES, TLE1 inhibited the pro-apoptotic action of AES (Fig. 8A and B).

Drosophila Groucho and human TLE proteins form homotetramers via association of the two α -helices in the Q domain (Chen et al., 1998). Heteromerization has been found to be essential for transcriptional repression by Groucho. Since AES has the Q domain, it is capable of heteromerizing with the longer TLE proteins (Pinto et al., 1996). Such an association between the TLE repressors proteins and AES would sequester the TLE proteins in an inactive form explaining the dominant-negative effect of AES. To examine whether AES and TLE1 interacts in intact, CHO cells were co-transfected with plasmids expressing AES and TLE1, and co-immunoprecipitation experiment was performed. As shown in Fig. 8C, immunoprecipitation of AES also pulled down TLE1.

DISCUSSION

In this study, we used the yeast two-hybrid system to identify novel proteins that interact with HDRP and this screen led to the isolation of AES, a member of the Groucho family of proteins. Interaction between AES and HDRP was confirmed using *in vitro* binding assays and in intact cells using co-immunoprecipitation and colocalization studies. Our studies identify AES as a new interacting partner of HDRP.

Another interesting finding of our study is the demonstration that AES has pro-apoptotic activity in neurons. Most attention on TLE family of proteins has been placed on the longer members, which have been reported to regulate a variety of developmental processes including neurodevelopment (Chen et al., 2000; Courey et al., 2001; Gasperowicz et al., 2005; Buscarlet et al., 2007). In comparison, virtually nothing is known about the role of AES during development or in the adult. Like some of its larger counterparts, AES is expressed ubiquitously (Mallo et al., 1995). Mice lacking AES have been generated. A large proportion of these mice are growth retarded during the first few weeks of life and display reduced Indian hedgehog signaling and skeletal abnormalities (Mallo et al., 1995; Wang et al., 2002). On the other hand, transgenic mice overexpressing AES die in utero (Allen et al., 2006). Analysis of lines expressing lower (sub-lethal levels) of the AES transgene has not revealed obvious abnormalities (Allen et al., 2006). Characterization of the brain or of other organs and tissues in these mice has not been reported (Allen et al., 2006).

What is the significance of the interaction between AES and HDRP to the regulation of neuronal survival? Our results indicate that the pro-apoptotic activity of AES is kept in check by HDRP through direct physical association. In neurons primed to die by LK treatment, there is a marked reduction in HDRP expression. This may permit AES to promote neuronal death unhindered by HDRP. Reestablishment of elevated HDRP levels via transfection results in the re-sequestration of AES inhibiting its pro-apoptotic action.

How does AES induce neuronal death? Tetsuka and colleagues (Tetsuka et al., 2000) have reported that AES interacts with NF- κ B protein p65 /RelA and represses its activity. It is well established that NF- κ B plays a pivotal role in the maintenance of neuronal survival both *in vitro* and *in vivo* (Mattson et al., 2000, 2001). A number of investigators have demonstrated that overexpression of p65 can protect against neuronal death induced by a variety of apoptotic stimuli including LK treatment (Daily et al., 2001; Kaltschmidt et al., 1999; Kovacs et al., 2004; Koulich et al., 2001; Piccioli et al., 2001;). It was possible that the interaction of AES with p65 interferes with the ability of NF- κ B to maintain neuronal survival. However, we have not been able to detect interaction between AES and p65 in co-immunoprecipitation experiments (data not shown) arguing against inhibition of p65 as being the mechanism by which AES promotes neuronal death.

Another possibility is that in neurons induced to die, the newly produced AES acts in a dominant-negative manner by replacing another TLE protein within an anti-apoptotic protein complex, rendering the protein complex unable to maintain cell survival. This may be facilitated by a reduction in the expression of the anti-apoptotic TLE within the protective complex and/or by the ability of AES to heteromerize with the longer TLE proteins. In healthy neurons and when overexpressed in neurons treated with LK, binding of HDRP to AES may sequester it from the anti-apoptotic TLE protein-containing complex, preventing cell death. Consistent with this possibility is our observation that HDRP and AES colocalize within the nuclei of neurons when co-expressed and that the two proteins co-immunoprecipitate when pulled down from overexpressed cell line lysates. Evidence that AES functions dominant-negatively in other biological systems has been reported by different investigators (Allen et al., 2006; Jan et al., 2004; Ren et al., 1999; Roose et al., 1998). In CHO cells in which AES overexpression causes apoptosis, it was found that the ability of AES to promote cell death is antagonized by the co-expression of TLE1 (Jan et al., 2004). Thus, TLE1 might represent the survival-promoting TLE that is antagonized by AES in neurons. In support of this possibility is our finding that TLE1 expression is reduced in neurons primed to die and that the overexpression of TLE1 in neurons prevents LK-induced apoptosis. Moreover, we also see that AES and TLE1 interact in intact cells. The development of lung tumors in mice overexpressing TLE1 (Allen et al., 2006) suggests that TLE1 has a similar anti-apoptotic function in other cell types too. Another pro-survival candidate member in CGNs could be TLE3. The expression of TLE3 was reported to be stimulated in neurons by depolarization (KCl) and treatment with forskolin (Feldman et al., 2000), two stimuli known to promote survival of CGNs and other neuronal types.

In summary, we report that AES has pro-apoptotic activity in neurons while TLE1 is protective. Our results indicate that the pro-apoptotic activity of AES is inhibited by HDRP via direct interaction. Although a number of proteins have been identified in recent years that interact with HDRP and other members of the HDAC family, this is the first identification of AES as an HDAC-interacting protein. Our previous work indicated that HDRP-mediated neuroprotection involves inhibition of c-jun. (Morrison et al., 2006). Indeed, HDRP represses histone acetylation at the c-jun gene promoter through the recruitment of HDAC1. We show that another mechanism by which HDRP prevents neuronal death is by inhibiting the pro-apoptotic activity of AES. It is conceivable that these two mechanisms are not mutually exclusive and that a relationship exists between AES and the stimulation of c-jun expression

and activity. For example, it is possible that HDRP is recruited to transcription regulatory complexes within the c-jun promoter by AES. Further work is necessary to clarify these issues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

This work was supported by funds from the National Institute of Neurological Diseases and Stroke (NS40408) to SRD.

ABBREVIATIONS

Grg5, Groucho-related protein-5; AES, Amino-terminal of Enhancer of Split; HDACs, Histone deacetylases; TLE, Transducin-like Enhancer of split; CGN, Cerebellar granule neuron; IP, Immunoprecipitation assay; GFP, Green fluorescent protein; HA, Hemagglutinin; NLS, Nuclear localization signal; RT-PCR, Reverse transcription-polymerase chain reaction.

REFERENCES

- Allen T, v. T. M, Iyengar P, et al. Grg1 acts as a lung-specific oncogene in a transgenic mouse model. *Cancer Res* 2006;66:1294–1301. [PubMed: 16452182]
- Borodezt K, D'Mello SR. Decreased expression of the metabotropic glutamate receptor-4 gene is associated with neuronal apoptosis. *J. Neurosci. Res* 1998;53:531–541. [PubMed: 9726424]
- Buscarlet M, Stifani S. The 'Marx' of Groucho on development and disease. *Trends Cell Biol* 2007;17:353–361. [PubMed: 17643306]
- Chen G, Courey AJ. Groucho/TLE family proteins and transcriptional repression. *Gene* 2000;249:1–16. [PubMed: 10831834]
- Chen G, P. N, Courey AJ, et al. A role for Groucho tetramerization in transcriptional repression. *Mol. Cell Biol* 1998;18:7259–7268. [PubMed: 9819412]
- Chin PC, L. L, Morrison BE, et al. The c-Raf inhibitor GW5074 provides neuroprotection in vitro and in an animal model of neurodegeneration through a MEK/ERK and Akt-independent mechanism. *J Neurochem* 2004;90:595–608. [PubMed: 15255937]
- Courey AJ, Jia S. Transcriptional repression: the long and the short of it. *Genes Dev* 2001;15:2786–96. [PubMed: 11691830]
- D'Mello SR, Chin PC. Treating neurodegenerative conditions through the understanding of neuronal apoptosis. *Curr Drug Targets CNS Neurol Disord* 2005;4:3–23. [PubMed: 15723610]
- D'Mello SR, Galli C, et al. Induction of apoptosis in cerebellar granule neurons by low potassium: inhibition of death by insulin-like growth factor I and cAMP. *Proc Natl Acad Sci U S A* 1993;90:10989–93. [PubMed: 8248201]
- Daily D, Vlamis-Gardikas A, et al. Glutaredoxin protects cerebellar granule neurons from dopamine-induced apoptosis by activating NF-kappa B via Ref-1. *J Biol Chem* 2001;276:1335–44. [PubMed: 11035035]
- de Ruijter AJ, van Gennip AH, et al. Histone deacetylases (HDACs): characterization of the classical HDAC family. *Biochem J* 2003;370:737–49. [PubMed: 12429021]
- Feldman JD, Vician L, et al. rTLE3, a newly identified transducin-like enhancer of split, is induced by depolarization in brain. *J Neurochem* 2000;74:1838–47. [PubMed: 10800926]
- Galli C, Meucci O, Scorziello A, Werge TM, Calissano P, Schettini G. Apoptosis in cerebellar granule cells is blocked by high KCl, forskolin, and IGF-1 through distinct mechanisms of action: the involvement of intracellular calcium and RNA synthesis. *J Neurosci* 1995;15:1172–1179. [PubMed: 7532699]
- Gasperowicz M, Otto F. Mammalian Groucho homologs: redundancy or specificity? *J Cell Biochem* 2005;95:670–687. [PubMed: 15861397]
- Hara MR, Snyder SH. Cell Signaling and Neuronal Death. *Annu Rev Pharmacol Toxicol*. 2006

- Jan Y, Matter M, et al. A mitochondrial protein, Bit1, mediates apoptosis regulated by integrins and Groucho/TLE corepressors. *Cell* 2004;116:751–62. [PubMed: 15006356]
- Kaltschmidt B, Uherek M, et al. Inhibition of NF-kappaB potentiates amyloid beta-mediated neuronal apoptosis. *Proc Natl Acad Sci U S A* 1999;96:9409–14. [PubMed: 10430956]
- Koulich E, Nguyen T, et al. NF-kappaB is involved in the survival of cerebellar granule neurons: association of IkappaBbeta [correction of Ikappabeta] phosphorylation with cell survival. *J Neurochem* 2001;76:1188–98. [PubMed: 11181838]
- Kovacs AD, Chakraborty-Sett S, et al. Mechanism of NF-kappaB inactivation induced by survival signal withdrawal in cerebellar granule neurons. *Eur J Neurosci* 2004;20:345–52. [PubMed: 15233744]
- Mallo M, Gendron-Maguire M, et al. Protein characterization and targeted disruption of Grg, a mouse gene related to the groucho transcript of the *Drosophila* Enhancer of split complex. *Dev Dyn* 1995;204:338–47. [PubMed: 8573724]
- Mattson MP, Camandola S. NF-kappaB in neuronal plasticity and neurodegenerative disorders. *J Clin Invest* 2001;107:247–54. [PubMed: 11160145]
- Mattson MP, Culmsee C, et al. Roles of nuclear factor kappaB in neuronal survival and plasticity. *J Neurochem* 2000;74:443–56. [PubMed: 10646495]
- Morrison BE, Majdzadeh N, et al. Neuroprotection by histone deacetylase-related protein. *Mol Cell Biol* 2006;26:3550–64. [PubMed: 16611996]
- Nardi N, Avidan G, Daily D, Zilkha-Falb R, Barzilai A. Biochemical and temporal analysis of events associated with apoptosis induced by lowering the extracellular potassium concentration in mouse cerebellar granule neurons. *J. Neurochem* 1997;68:750–759. [PubMed: 9003066]
- Piccoli P, Porcile C, et al. Inhibition of nuclear factor-kappaB activation induces apoptosis in cerebellar granule cells. *J Neurosci Res* 2001;66:1064–73. [PubMed: 11746438]
- Pinto M, L. C. Products of the grg (Groucho-related gene) family can dimerize through the amino-terminal Q domain. *J Biol Chem* 1996;271:33026–33031. [PubMed: 8955148]
- Ren B, Chee KJ, et al. PRDI-BF1/Blimp-1 repression is mediated by corepressors of the Groucho family of proteins. *Genes Dev* 1999;13:125–37. [PubMed: 9887105]
- Roose J, Molenaar M, et al. The *Xenopus* Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors. *Nature* 1998;395:608–12. [PubMed: 9783587]
- Schulz JB, Weller M, Klockgether T. Potassium deprivation-induced apoptosis in cerebellar granule neurons: A sequential requirement for new mRNA and protein synthesis, ICE-like protease activity, and free radicals. *J. Neurosci. Res* 1996;16:4696–4706.
- Tetsuka T, Uranishi H, et al. Inhibition of nuclear factor-kappaB-mediated transcription by association with the amino-terminal enhancer of split, a Groucho-related protein lacking WD40 repeats. *J Biol Chem* 2000;275:4383–90. [PubMed: 10660609]
- Verdin E, Dequiedt F, et al. Class II histone deacetylases: versatile regulators. *Trends Genet* 2003;19:286–93. [PubMed: 12711221]
- Wang WF, Wang YG, et al. Growth defect in Grg5 null mice is associated with reduced Ihh signaling in growth plates. *Dev Dyn* 2002;224:79–89. [PubMed: 11984876]
- Yang XJ, Gregoire S. Class II histone deacetylases: from sequence to function, regulation, and clinical implication. *Mol Cell Biol* 2005;25:2873–84. [PubMed: 15798178]

Figure 1. Mapping of the AES - HDRP interaction domain by yeast two hybrid assay

A). Schematic illustration of AES and fragments encoding AES sequences isolated in yeast two hybrid screening. #6, #118, #261 and #271 correspond to cDNA inserts of four positive clones isolated from the yeast two-hybrid screening. B). the different bait HDRP constructs used in two-hybrid assays to map the region involved in associating with AES are shown on the left. The ability of these constructs to interact with prey molecules, AES (1–197) and allow growth of yeast on selective plates is shown on the right. These studies map the domain of HDRP required for interaction with AES to the region spanning residues spanning 178–303. Full length HDAC1 cloned in bait vector pGBKT7 was co-transformed with the prey plasmid pACT-AES to test interaction. Empty bait vector pGBKT7 was co-transformed with the prey plasmids, and the empty prey plasmid pACT2 was co-transformed with the bait expression plasmid as additional control.

Figure 2. HDRP associates with AES *in vitro*

A). AES- GST fusion proteins were conjugated to glutathione sepharose beads and incubated with ³⁵S-methionine-labeled HDRP. Bound proteins were eluted for SDS-PAGE and autoradiography. ³⁵S –labeled luciferase was used as negative control. Input lanes represent 10% of [³⁵S] methionine-labeled proteins used for binding assays and serve as a reference marker. B). HDRP 1–343aa, 1–149aa, 178–591aa – GST proteins were conjugated to glutathione sepharose beads and incubated with ³⁵S-methionine-labeled AES. Associated proteins were resolved by SDS-PAGE followed by autoradiography. ³⁵S –labeled luciferase was used as negative control. Constructs used in the study are summarized in both A and B.

Figure 3. Subcellular colocalization and co-immunoprecipitation of AES and HDRP

A). CHO cells were cotransfected with plasmids expressing Xpress/His-AES and Flag-HDRP. The subcellular localization of these tagged proteins was visualized using primary antibody (Flag or His antibody) and secondary antibody (Texas-red or FITC-conjugated antibody) respectively. Chromatin was stained with DAPI to visualize nuclei. B). Expression plasmids for Flag-HDRP and Xpress/His-AES were transfected into CHO cells, and extracts were prepared for immunoprecipitation on A/G plus agarose beads. Equal amounts of protein were incubated overnight with 1 μ g of anti-Flag primary antibody at 4°C. Precipitated proteins were eluted and analyzed by Western blotting using anti-Xpress and whole cell lysates (WCL) were analyzed by Western blotting using anti-Xpress and anti-Flag antibodies as input.

Figure 4. Expression of HDRP, AES and TLE1 during neuronal apoptosis

A). Cultured CGNs were treated with HK or LK medium for 3 h. RNA levels for indicated genes were determined by RT-PCR using specific primers. The level of β -actin was measured to ensure equal amount of cDNA. B). HDRP, AES, C-Jun protein expression in neuronal cultures subjected to time-course treatment with HK or LK. Lysates were obtained from cultures treated with HK or LK for 3 and 6 h. Western blotting was performed by using HDRP, AES, C-Jun primary antibody, and the membrane was probed with tubulin antibody to confirm equal protein loading.

Figure 5. AES has pro-apoptotic activity in neurons

A). Neuronal cultures were transfected with a plasmid encoding Xpress/His-AES. The cultures were then treated with HK, LK or LK medium supplemented with 1 μ M GW5074 medium for 24 h and the proportion of positively-transfected neurons were examined by immunocytochemistry with primary antibody against Xpress. B). Proportion of cells that were apoptotic, as judged by condensed or fragmented nuclei based on DAPI-staining was then determined (data indicates mean, $\pm$ S.D. n = 3, * indicates that the p-value < 0.05 using t-test compared with GFP transfected CGNs in the same condition).

Figure 6. HDRP inhibited the ability of AES to promote neuronal death

A). CGN cultures were cotransfected with plasmids encoding Xpress/His-AES and Flag-HDRP and the cultures were then treated with HK or LK medium for 24 h. The proportion of positively-transfected neurons were examined by immunocytochemistry with primary antibody against His and Flag. B). Proportion of cells that were apoptotic, as judged by condensed or fragmented nuclei based on DAPI-staining was then determined (data indicates mean, $\pm$ S.D. n = 3, * indicates that the p-value < 0.05 using t-test compared with GFP transfected CGNs in the same condition).

Figure 7. TLE1 has an anti-apoptotic effect in neurons

A). Plasmid encoding Flag-TLE1 was transfected to CGN cultures. Cultures were then treated with HK, LK medium for 24 h. The proportion of positively-transfected neurons were examined by immunocytochemistry with primary antibody against Flag. B). The proportion of cells that were apoptotic, as judged by condensed or fragmented nuclei based on DAPI-staining was then determined (data indicates mean, $\pm$ S.D. n = 3. * indicates that the p-value < 0.05 using t-test compared with GFP transfected CGNs in LK).

Figure 8. AES binds TLE1 *in vivo*, and TLE1 blocks apoptosis induced by AES in CGN cells
 A). CGN cultures were cotransfected with Flag-TLE1 and Xpress/His-AES. Cultures were then treated with HK, LK medium for 24 h. The subcellular localization of these tagged proteins was visualized using Flag or His antibody and a Texas-red and FITC-conjugated secondary antibody, respectively. Chromatin was stained with DAPI to visualize nuclei. B). Xpress/His-AES and Flag-TLE1 was cotransfected to CGNs and then the cultures were treated with HK or LK medium for 24 h. The proportion of positively-transfected neurons were examined by immunocytochemistry with an antibody against His and Flag. The proportion of cells that were apoptotic, as judged by condensed or fragmented nuclei based on DAPI-staining was then determined (data indicates mean, $\pm$ S.D. $n = 3$, * indicates that the p -value < 0.05 using t-test compared with GFP transfected CGNs at the same condition.) C). Expression plasmids for Flag-TLE1 and Xpress/His-AES were transfected into HEK293T cells, and extracts were prepared for immunoprecipitation on A/G plus agarose beads. Precipitated proteins were eluted and analyzed by Western blotting with anti-Flag antibodies. Whole cell lysates were analyzed by Western blotting using anti-Xpress and anti-Flag antibodies as input.

Figure 9. Models explaining relationships between HDRP, AES, and TLE1 in the regulation of neuronal survival

A). In healthy neurons maintained in HK medium, HDRP sequesters AES through direct interaction and thus inhibits its pro-apoptotic action. When neurons are switched to LK medium, the level of HDRP is reduced. This allows AES to promote apoptosis. The overexpression of HDRP by transfection with HDRP prevents neuronal death by neutralizing the pro-apoptotic effect of AES. B). TLE1 is part of an anti-apoptotic transcriptional complex in neurons maintained in HK medium. In neurons primed to die by LK treatment, TLE1 expression is down regulated. It is replaced in the complex with AES, which has a dominant-negative effect resulting in the inactivation of the transcriptional complex. AES might also sequester TLE1 by directly interacting with it.