RNA Interference Reveals a Requirement for Myocyte Enhancer Factor 2A in Activity-dependent Neuronal Survival*

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RNA interference (RNAi) provides a powerful method of gene silencing in eukaryotic cells, including proliferating mammalian cells. However, the utility of RNAi as a method of gene knockdown in primary postmitotic mammalian neurons remained unknown. Here, we asked if RNAi might be utilized to allow the assessment of the biological function of a specific gene in the nervous system. We employed a U6 promoter-driven DNA template approach to induce hairpin RNA-triggered RNAi to characterize the role of the transcription factor myocyte enhancer factor 2A (MEF2A) in the neuronal activity-dependent survival of granule neurons of the developing rat cerebellum. We found that the expression of MEF2A hairpin RNAs leads to the efficient and specific inhibition of endogenous MEF2A protein expression in primary cerebellar granule neurons. We also found that RNAi of MEF2A reduces significantly MEF2-dependent granule neuron survival. Taken together, our RNAi experiments have revealed that MEF2A plays a critical role in activity-dependent neuronal survival. In addition, our findings indicate that RNAi does operate in postmitotic mammalian neurons and thus offers a rapid genetic method of studying gene function in the development and function of the mammalian nervous system.

RNA interference (RNAi)1 is the process of gene silencing whereby double-stranded RNA induces the homology-dependent degradation of its cognate mRNA. Since its discovery in Caenorhabditis elegans (1), RNAi has become a powerful and widely used tool for the analysis of gene function in many systems, including fungi, plants, and Drosophila (2, 3). Biochemical studies have revealed that cells contain an evolutionarily conserved RNAi machinery that triggers the cleavage of double-stranded RNAs into 21- or 22-nucleotide small interfering RNAs (siRNAs). The siRNAs then hybridize to their cognate mRNA, thereby inducing the specific degradation of the target mRNA.

An ultimate goal of RNAi studies is to knock down mammalian gene expression in the intact organism in a tissue-specific manner. Recently, the introduction of synthetic siRNAs in mammalian cells has been demonstrated to induce specific gene silencing (4). However, although RNAi has been used successfully in a number of cell lines (4–13), it remained unknown whether RNAi-mediated gene silencing operates in primary postmitotic cells, a question that is of particular significance to studies of the nervous system. To begin to address this issue, it is essential to assess whether the RNAi machinery functions efficiently in postmitotic mammalian neurons.

In this study, we addressed the question of the utility of RNAi in mammalian neurons by specifically disrupting the endogenous expression of a transcription factor in primary granule neurons cultured from the developing rat cerebellum. In previous studies employing dominant negative strategies, members of the myocyte enhancer factor 2 (MEF2) family of proteins have been suggested to contribute to neuronal survival (14–17). However, because a dominant interfering form of a transcription factor has the potential to block the activity of all proteins within the family of the transcription factor of interest, this approach does not allow the assessment of the function of a specific family member. The MEF2 transcription factors MEF2A, MEF2B, MEF2C, and MEF2D are expressed in the central nervous system (18, 19). Among the MEF2 proteins, MEF2A is highly expressed in cerebellar granule neurons and is regulated posttranslationally in these neurons upon neuronal activity (14, 15). However, the specific role of MEF2A in activity-dependent granule neuron survival remained to be characterized.

We sought to determine the role of MEF2A in activity-dependent granule neuron survival by the RNAi method. We employed a recently developed DNA vector-based technique (11) in which siRNAs are processed from a short RNA hairpin (hpRNA) transcribed under the control of the polymerase III U6 promoter. We found that the expression of MEF2A hpRNAs dramatically reduces the expression of endogenous MEF2A protein in primary cerebellar granule neurons and thereby inhibits MEF2-dependent transcription and granule neuron survival. Our findings support two conclusions. 1) RNAi can be employed to suppress gene expression and function in primary postmitotic neurons of the developing mammalian central nervous system. 2) Among the known MEF2 transcription factors, MEF2A is required for activity-dependent cerebellar granule neuron survival.

EXPERIMENTAL PROCEDURES

Plasmid Design—The U6/mef2a and U6/nd1 RNAi constructs were designed as described by Sui et al. (11). Briefly, 22-nucleotide-long inverted repeats, separated by a 6-nucleotide linker, were inserted downstream of the U6 promoter. The transcribed RNA thus comprised...

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1 The abbreviations used are: RNAi, RNA interference; siRNA, small interfering RNA; MEF2, myocyte enhancer factor 2; MRE, MEF2 response element; hpRNA, hairpin RNA; DIV, days in vitro; cdk2, cyclin-dependent kinase 2.

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were transfected with the U6, U6/mef2a, or U6/nd1 RNAi construct (100 nM MRE of three MEF2 response elements placed upstream of the firefly luciferase gene under the control of the constitutively active cytomegalovirus (CMV) promoter). After 2 days in vitro (DIV), granule neurons were transfected using a modified calcium phosphate method (25), i.e., cells were fixed with 4% paraformaldehyde and subjected to indirect B-galactosidase expression and the integrity of the nucleus as determined by the DNA dye bisbenzimide (Hoechst 33258).

Cerebellar Cultures—Granule neurons were isolated from a P6 rat cerebellum and plated as described (20). Neuronal activity was mimicked by providing elevated levels of extracellular potassium chloride that induced membrane depolarization and consequent activation of the voltage-sensitive calcium channels (21–24). After 2 days in vitro (DIV), granule neurons were transfected with 105 cerebellar granule cells and left on the cells for 15 min. In the experiments shown in Figs. 3, A and B, and Fig. 4B, cultures were transfected with 1–2 μg of the U6, U6/mef2a, or U6/nd1 vector together with a mouse monoclonal antibody to β-galactosidase (dilution 1:500, Promega). The MEF2A signal was detected with a goat anti-MEF2A antibody (Fig. 2A) or an anti-FLAG mouse monoclonal antibody (Sigma, Fig. 2B) using LipofectAMINE 2000 (Invitrogen). Cells were harvested 72 h after transfection, and 20 μg of total protein (measured by the Bradford assay) were loaded on a 10% polyacrylamide gel and analyzed by Western analysis using a rabbit anti-MEF2A antibody (Fig. 2A) or an anti-FLAG mouse monoclonal antibody (Sigma, Fig. 2B).

Western Analysis—Neuro2A cells were transfected with 1 μg of an expression vector encoding MEF2A (Fig. 2A), FLAG-tagged NeuroD (Fig. 2B), or the pCDNA3 control vector (Fig. 2, A and B) using LipofectAMINE 2000 (Invitrogen). Cells were harvested 72 h after transfection, and 20 μg of total protein (measured by the Bradford assay) were loaded on a 10% polyacrylamide gel and analyzed by Western analysis using a rabbit anti-MEF2A antibody (Fig. 2A) or an anti-FLAG mouse monoclonal antibody (Sigma, Fig. 2B).

RESULTS AND DISCUSSION

To determine whether RNAi might be induced in primary mammalian neurons, we used a DNA vector-based RNAi method to target the transcription factor MEF2A in cerebellar granule neurons. To assess whether vector-based RNAi can mediate specific gene silencing in primary neurons by way of comparison with RNAi of MEF2A, we also targeted the transcription factor NeuroD. Both MEF2A and NeuroD are highly expressed in cerebellar granule neurons and are therefore easily detected immunocytochemically (18, 19, 26, 27).

The U6/mef2a and U6/nd1 constructs (Fig. 1) were designed as described in Sui et al. (11) and encode two hpRNAs that target the MEF2A and NeuroD mRNA, respectively. The 22-nucleotide-long target sequences were chosen because they have no significant homology with other known genes, including the other members of the MEF2 and NeuroD families (Fig. 1C). We first examined whether transcribed hpRNAs specifically reduce the expression of the cognate gene when expressed together with a mouse monoclonal antibody to β-galactosidase (dilution 1:500, Promega). The MEF2A signal was detected with a goat anti-MEF2A antibody conjugated to Cy3. For the experiment shown in Fig. 3B, cells were fixed in 100% methanol and subjected to indirect immunofluorescence with a goat anti-NeuroD antibody (Santa Cruz Biotechnology, dilution 1:50) together with the mouse monoclonal antibody to β-galactosidase. The NeuroD signal was detected with a donkey anti-goat secondary antibody conjugated to Cy3. The β-galactosidase signal was detected with a goat (Fig. 3A) or donkey (Fig. 3B) anti-mouse secondary antibody conjugated to Cy2.

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Western Analysis—Neuro2A cells were transfected with 1 μg of the control vector (U6), U6/mef2a, or U6/nd1 RNAi construct together with 1 μg of an expression vector encoding MEF2A (Fig. 2A), FLAG-tagged NeuroD (Fig. 2B), or the pCDNA3 control vector (Fig. 2, A and B) using LipofectAMINE 2000 (Invitrogen). Cells were harvested 72 h after transfection, and 20 μg of total protein (measured by the Bradford assay) were loaded on a 10% polyacrylamide gel and analyzed by Western analysis using a rabbit anti-MEF2A antibody (Fig. 2A) or an anti-FLAG mouse monoclonal antibody (Sigma, Fig. 2B).

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in a neuronal cell line. Neuro2A cells were transfected with the control vector (U6), U6/mef2a, or the U6/nd1 RNAi construct together with an expression vector encoding MEF2A (Fig. 2A) or FLAG-tagged NeuroD (Fig. 2B). The expression of MEF2A and NeuroD in transfected Neuro2A cells was measured 3 days after transfection by Western analysis using a rabbit antibody that recognizes MEF2A or a monoclonal anti-FLAG antibody to detect FLAG-NeuroD. The expression of MEF2A hpRNA in Neuro2A cells led to a dramatic reduction in the level of coexpressed MEF2A protein (Fig. 2A, lane 4) but had no effect on the expression of NeuroD (Fig. 2B, lane 4). Similarly, the expression of NeuroD hpRNA in Neuro2A cells resulted in the reduction of NeuroD expression to background levels (Fig. 2B, lane 6) but did not alter MEF2A expression (Fig. 2A, lane 6). These results indicate that the MEF2A and NeuroD hpRNAs block, respectively, the expression of exogenous MEF2A and NeuroD, effectively and specifically.

We then determined the utility of vector-based RNAi to knock-down endogenous gene expression in primary cultures of cerebellar granule neurons. Cerebellar granule neurons were prepared from P6 rat pups and transfected at 2 DIV using a modified calcium phosphate transfection method (25) with the U6, U6/mef2a, or U6/nd1 RNAi construct together with an expression plasmid encoding β-galactosidase. Three days after transfection, cultures were subjected to indirect immunofluorescence using a rabbit antibody to MEF2A or a goat antibody to NeuroD and a mouse monoclonal antibody to β-galactosidase. β-galactosidase immunoactivity was used to identify transfected neurons. In untransfected granule neurons and neurons transfected with the U6/control vector, robust endogenous MEF2A or NeuroD immunoreactivity was detected in the nucleus (Fig. 3, A and B). By contrast, in neurons transfected with the U6/mef2a construct, endogenous MEF2A immunoreactivity was barely detected (Fig. 3A, white arrowhead). Similarly, transfection of granule neurons with the U6/nd1 construct suppressed effectively the expression of endogenous NeuroD (Fig. 3B, white arrowhead).

To assess the efficiency of vector-based RNAi silencing in the granule neurons, we quantitated the percentage of transfected neurons that contained a high level of expression of endogenous MEF2A or NeuroD. At least 150 neurons per condition were scored in three independent experiments (Fig. 3C). Whereas 90% of vector-transfected granule neurons contained high endogenous MEF2A immunoreactivity, only 30% of the MEF2A hpRNA-expressing granule neurons displayed high endogenous MEF2A immunoreactivity. In the case of the NeuroD RNAi, the difference in endogenous NeuroD immunoreactivity between vector-transfected and NeuroD hpRNA-expressing granule neurons was even more dramatic (Fig. 3D). Importantly, the expression of NeuroD hpRNA had no effect on endogenous MEF2A expression in granule neurons (Fig. 3C).

The finding that RNAi of MEF2A triggers the knock-down of MEF2A expression in granule neurons led us to examine the biological role of endogenous MEF2A in cerebellar granule neurons. Members of the MEF2 family of transcription factors mediate membrane depolarization-induced transcription in neurons via the MEF2-response element (MRE) that is found within the promoter of MEF2-responsive genes (14, 15). The inhibition of MEF2-dependent transcription in membrane-depolarized granule neurons by dominant interfering forms of MEF2 triggers apoptosis, suggesting that members of the MEF2 family mediate activity-dependent survival of granule neurons (14, 15). However the specific role of the family member MEF2A in activity-induced MRE-dependent transcription and neuronal survival remained to be elucidated.

To characterize the biological role of MEF2A in cerebellar granule neurons, we first determined the effect of MEF2A hpRNA on MRE-mediated transcription in granule neurons (Fig. 4A). Granule neuron cultures (P6 + 2 DIV) were transfected with a MEF2-specific reporter containing three MREs upstream of the firefly luciferase reporter gene (3×MRE fluc) together with a Renilla luciferase reporter gene to serve as a control for transfection efficiency. Cerebellar granule neuron cultures were then exposed to elevated levels of potassium chloride that induce membrane depolarization and the consequent activation of voltage-sensitive calcium channels (23). Two days after transfection, the expression of the 3×MRE fluc reporter gene was measured. The activity of the 3×MRE fluc reporter gene was significantly reduced in neurons transfected with the MEF2A RNAi construct as compared with neurons transfected with the U6 vector (Fig. 4A). By contrast, the expression of the NeuroD hpRNA failed to reduce the activity of the MRE luciferase reporter gene. These results suggest that MEF2A mediates MRE-dependent transcription in membrane-depolarized cerebellar granule neurons.

In a second set of experiments, we determined the effect of MEF2A RNAi on the survival of cerebellar granule neurons upon membrane depolarization. At 2 days after transfection with the RNAi constructs, at the time that luciferase assays were carried out, there was little if any difference in survival between vector control and U6/mef2a-transfected neurons.
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Fig. 3. Knock-down of endogenous MEF2A and NeuroD in cerebellar granule neurons. Cerebellar granule neurons were transfected with the U6 control vector, the U6/mef2a (panel A), or the U6/nd1 (panel B) vector together with the β-galactosidase expression vector. After 5 days in full medium, transfected cultures were fixed and subjected to indirect immunofluorescence. Left column, anti-β-galactosidase (anti-beta-gal) and anti-MEF2A (panel A) or anti-NeuroD (panel B) immunofluorescence. Middle column, anti-MEF2A (panel A) or anti-NeuroD (panel B) immunofluorescence. Right column, Hoechst 33258 staining. C and D, quantitation of results in panels A and B, respectively. The expression of MEF2A hpRNAs but not NeuroD hpRNAs reduced significantly the expression of endogenous MEF2A protein in granule neurons (Fig. 4A, \( p < 0.0001, n = 3 \), analysis of variance). Similarly, the expression of NeuroD hpRNAs but not MEF2A hpRNAs reduced significantly the expression of endogenous NeuroD (Fig. 4B, \( p < 0.0001, n = 3 \), analysis of variance). In each condition, at least 150 neurons were counted.

Fig. 4. MEF2A RNAi inhibits the function of endogenous MEF2A in cerebellar granule neurons. A, dual-luciferase assays using the 3×MRE fluc reporter (see “Experimental Procedures”). The firefly luciferase values are shown relative to the Renilla luciferase values. In comparison to U6-transfected neurons, the expression of the MEF2A hpRNAs induced a 75% decrease in the MRE-mediated luciferase activity \( (p < 0.0005, n = 8, \) analysis of variance), whereas the expression of NeuroD hpRNAs had little effect on MRE-mediated transcription. B, survival of granule neurons transfected with the U6 control vector, the U6/mef2a, or the U6/cdk2 construct. Cells were transfected at 2 DIV and fixed 4 or 5 days later. MEF2A hpRNA-expressing granule neurons, as compared with U6/cdk2- or control vector-transfected granule neurons, underwent increased apoptosis at 4 and 5 days after transfection \( (p < 0.0005, n = 3, \) analysis of variance). There was no difference in the rate of apoptosis of untransfected neurons in cultures transfected with the U6 control vector, the U6/mef2a, or the U6/cdk2 construct.

(design not shown). However, we found that the expression of MEF2A hpRNA reduced significantly the survival of membrane-depolarized cerebellar granule neurons at 4 and 5 days after transfection (Fig. 4B). To rule out the possibility of a nonspecific toxic effect of MEF2A hpRNA on cell survival secondary to the production of hpRNA, we tested the effect of hpRNA targeting the unrelated gene cyclin-dependent kinase 2 (cdk2). The cdk2 hpRNA were shown to specifically reduce the expression of cdk2 \( (11, \) analysis of variance). By contrast with MEF2A hpRNA, the expression of cdk2 hpRNA in granule neurons had no detectable effect on activity-dependent granule neuron survival. Taken together, these results suggest that MEF2A is required for activity-dependent cerebellar granule neuron survival.

To sum up, we have found that the RNAi machinery functions efficiently in primary postmitotic mammalian neurons. As a result, the vector-based RNAi method allowed us to determine the specific function of the transcription factor MEF2A in cerebellar granule neurons. We found that knock-down of MEF2A suppresses activity-induced MRE-mediated transcription and granule neuron survival. Together, these results suggest that MEF2A plays a critical role in the development of cerebellar granule neurons. In future studies, knock-down of MEF2A in neurons should facilitate the identification of MEF2A-responsive genes that mediate activity-dependent neuronal survival.

Our findings have several implications. Because of its relative simplicity, the vector-based RNAi method should provide a rapid means of analysis of gene function in primary cultures of mammalian neurons. Because of the specificity of RNAi in reducing the expression of a target gene, one can assess the biological role of a particular member of a given protein family, a task that is often difficult to achieve by standard dominant negative approaches. Finally, because RNAi operates in primary postmitotic mammalian neurons, RNAi is likely to be of utility in the study of the nervous system in the intact mammal.
malian organism and thus precede or complement a gene knock-out strategy.

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