Cyclophilin A Is Required for Retinoic Acid-induced Neuronal Differentiation in p19 Cells*

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Stable transfectants with expression of small interfering RNA for targeting cyclophilin A (CypA) in p19 cells lose their potential for retinoic acid (RA)-induced neuronal differentiation but not Me2SO-induced mesodermal differentiation. This difference suggests that CypA is specifically required for the RA-induced neuronal pathway. In addition to the loss of RA-induced RA receptor β expression and retinoic acid response element (RARE)-binding activity, a dramatic reduction in RA-induced RARE-mediated luciferase activity in the CypA knockdown cell line suggests that CypA affects RARE-mediated regulation of gene expression. Silent mutation of target sequences confirms the specificity of RNA interference in p19 embryonal carcinoma cells. Collectively, our data reveal that a novel function of CypA is required in the processing of RA-induced neuronal differentiation in p19 embryonal carcinoma cells.

Cyclophilin A (CypA) is a housekeeping gene (1–3) that belongs to the immunophilin protein family. The remarkable evolutionary conservation and broad cellular and tissue distribution of this family of proteins suggest their fundamental importance in the cell, but the biological function of the core cyclophilin domain is unknown. CypA was first identified and purified from bovine spleen, based on its high affinity for the immunosuppressive drug cyclosporin A (4). CypA, similar to other cyclophilin family members, possesses enzymatic peptidylprolyl isomerase activity, which is essential to protein folding in vivo. Although CypA has a pivotal role in the immune response, it is not essential for mammalian cell viability (5). Recently, different aspects of the biological function of CypA have emerged. CypA binds to the human immunodeficiency virus type 1 Gag protein and is required for wild-type human immunodeficiency virus type 1 replication kinetics (6). CypA also promotes proper subcellular localization of Zpr1p, a zinc-finger-containing protein (7). Moreover, CypA regulates interleukin-2 tyrosine kinase activity (8) and has been implicated in neuronal cell growth and differentiation (9). These data provide a molecular basis for further understanding a role for CypA in cellular function.

Although CypA is widely expressed in many tissues, it is most concentrated in the brain and located primarily in neurons (10). Our recent data demonstrate that the nuclei of ganglia sensor neurons are particularly enriched with CypA (11), suggesting that CypA might be important for neuron cells. To test our hypothesis, we used siRNAs to block the expression of CypA in p19 embryonal carcinoma (EC) cells, which have been used as a model system to study neuronal differentiation. Here we demonstrate that the vector-based system produces siRNAs in p19 EC cells and results in specific and persistent knockdown of CypA in stable transfectants. These CypA knockdown cell lines are consequently unresponsive to retinoic acid (RA)-induced neuronal differentiation. Furthermore, we confirm that the observed knockdown phenotype is the result of silencing of the intended target, by using a rescue plasmid to restore RA-induced differentiation potential as wild-type p19 EC cells. These results clearly indicate that CypA is essential during RA-induced neuronal differentiation of p19 EC cells.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—PCR was used to generate the U6 promoter and two cloning sites, Sall and HindIII. The PCR product was subcloned into the BamHI and HindIII sites of pENT vector to generate the siRNA expression vector, pNeoRNAi (Fig. 1A). Target sites were selected from the mouse cyclophilin A sequence (GenBank™ accession number X52803). Each hairpin siRNA sequence contained a 5′ Sall cloning site followed by 20-mer of target sequences, a 4-mer loop sequence (TTTG), another 20-mer of complementary target sequence, the transcription terminator (TTTTT), and the 3′ HindIII cloning site. The full length of the sequences, which were synthesized in the forward and reverse directions and annealed to form double strand DNA, is approximately 70-mer. This double strand DNA was cloned into pNeoRNAi to form pNeoRNAi-S1 and pNeoRNAi-S3. To construct pcDNA3-CypA-wt, full-length cDNA for mouse CypA was amplified using reverse transcriptase PCR. The fragment was then subcloned into pcDNA3 (Invitrogen). pcDNA3-CypA-re was generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

**Cell Culture and Transfection**—Murine embryonal carcinoma cell line p19 cells were grown in minimum essential medium alpha supplemented with 7.5% bovine calf serum and 2.5% fetal bovine serum (Invitrogen). RA-induced and dimethyl sulfoxide (Me2SO)-induced differentiations of the pluripotent murine embryonal carcinoma p19 cell line were performed according to the method described by Gill et al. (12). Twenty micromgrams each of plasmid pNeoRNAi-S1 and pNeoRNAi-S3 were electroporated into 5 × 10^6 cells. G418 (300 μg/ml) was added to the medium the next day for selection of neomycin-resistant transfectants, which were then picked 2 weeks later and maintained in 24-well plates. Cells were cultured for an additional 2 weeks before harvesting for screening of CypA expression using Western blot analysis.

**Northern Blot Analyses**—To detect the expression of siRNAs, 30 μg of each sample was separated on a 10% polyacrylamide gel and electro-
transferred to nylon membranes. Radiolabeled 20-mer oligonucleotides of the sense target sequence were used as probes.

**Western Blot Analyses**—Whole cell lysates were electrophoresed and immunoblotted according to the protocol provided by Santa Cruz Biotechnology, Santa Cruz, CA.

**Immunofluorescent Staining**—Cells cultured in Lab-Tek chamber slides (Nalgene Nunc International, Naperville, IL) were fixed for 15 min at 25 °C in 10% phosphate-buffered formalin and permeabilized with 0.4% Triton X-100 in phosphate-buffered saline for 5 min at 25 °C. CypA was detected by indirect immunofluorescent staining with polyclonal anti-cyclophilin A (BIOMOL INTERNATIONAL, Plymouth Meeting, PA) followed by secondary antibody fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Santa Cruz Biotechnology). Mounted coverslips were viewed with a BX41 Olympus fluorescence microscope, and images were captured with an Olympus digital camera.

**Cell Counts**—Cells were counted using the trypan blue assay kit (ICN Biomedicals, Inc., Aurora, OH). Equal numbers of cells were cultured in 6-well plates. The cells were harvested at different time points and collected by centrifugation. The cell pellets were gently resuspended in 200 µl of medium. Ten microliters of resuspended cells were gently mixed with 10 µl of trypan blue (0.4% in phosphate-buffered saline) for 1 min, and the number of live cells was counted by microscopic examination.

**Luciferase Assay**—Cells were cultured in 12-well plates and transfected with 2 µg of pTk-Luc or pRARE-TK-Luc using LipofectAMINE 2000 (Invitrogen). Cells were treated with RA (5 × 10⁻⁷ M) 4 h post-transfection or left untreated for 24 h. Cell lysates were used for luciferase assays using the dual-luciferase reporter assay system (Promega, Madison, WI) according to the manufacturer’s protocol.

**Electrophoretic Mobility Shift Assay (EMSA)”—** EMSA were performed as described previously by Song et al. (13). An end-labeled (γ-³²P)ATP double-stranded oligonucleotide containing a single RA response element (RARE) consensus sequence (ac-2559, Santa Cruz Biotechnology) was used as a template. For a competition study, a 100-fold molar excess of unlabeled oligonucleotides was added to the reaction mixture prior to the addition of the radiolabeled probe. Mobility shift reactions were resolved on 4% nondenaturing polyacrylamide gels. Gels were then dried and exposed to x-ray film with an intensifying screen at −80 °C.

**RESULTS**

**Hairpin siRNAs Specifically Inhibit Expression of CypA**—Attempting to test the hypothesis that CypA plays an important role in neuron cell function, we used small interfering RNAs to silence CypA expression in p19 EC cells, which have been used as a model system to study neuronal differentiation. The siRNA vector, pNeoRNAi, with a neomycin selection marker facilitated the establishment of CypA knockdown cell lines by producing hairpin siRNAs in p19 EC cells (Fig. 1B).

Plasmids that express hairpin siRNAs were transfected into p19 EC cells, and G418 was used for the selection of neomycin-resistant transfected cell lines. The selected transfected cells were screened for knockdown CypA expression by Western blot analysis, using anti-CypA antibodies (BIOMOL). CypA expression in transfectants S1-7 and S3-2 was significantly inhibited (Fig. 1C). Transfectants S1-1, S1-2, S3-1, and S3-6 were inhibited to a lesser extent (Fig. 1C). A neo-resistant transfected demonstrated normal levels of CypA (Fig. 1C).

To determine whether the inhibition of CypA resulted from the expression of siRNAs, we examined siRNA expression us-
Fig. 3. CypA is essential for RA-induced neuronal differentiation of p19 cells. A, growth rates of knockdown cell lines are shown with or without RA treatment. Trypan blue staining was used to count the living cells. The data show the average from three independent assays. B, RA induces neurite formation in wild-type p19 cells. Phase-contrast microscopy shows that RA induces neurite formation in RA-induced terminal neuronal differentiation but not in the S1-7 CypA knockdown cell line. C, CypA was involved in the RA-induced differentiation of p19 cells. Neur-on-specific β-tubulin III was used as a differentiation marker for the Western blotting analysis. The knockdown cell lines, the wild-type cells, and the empty vector-transfected cell line were treated with or without RA. Membranes stained with Coomassie Brilliant Blue were used as controls in the Western blotting analyses. D, neurofilament expression is induced in RA-induced neuronal differentiation. pNeoRNAi empty vector-transfected p19 cells and the CypA knockdown cell line S1-7 were processed for differentiation of neurons in the presence or absence of RA treatment. Cell lysates were prepared for Western blot analysis by probing with anti-neurofilament (68 kDa) polyclonal antibody (AB1983, Chemicon International, Temecula, CA). Membranes stained with Coomassie Brilliant Blue were used as controls with the Western blot analyses. E, GATA4 shows increased expression in the Me2SO-induced mesodermal differentiation. The pNeoRNAi empty vector-transfected p19 cells and the CypA knockdown cell line S1-7 were processed for mesodermal differentiation in the presence or absence of Me2SO treatment. Cell lysates were prepared for Western blot analysis by probing with anti-GATA4 polyclonal antibody (sc-9053, Santa Cruz Biotechnology). Membranes stained with Coomassie Brilliant Blue were used as controls with the Western blot analyses.

CypA Is Required for RA-induced Neuronal Differentiation of p19 Cells—The cell growth rates of the established cell lines S1-7 and S3-2 were noticeably faster than that of wild-type p19 EC cells. We therefore counted cell numbers at different time points under normal cell culture conditions in the absence or presence of RA treatment. In the absence of RA, growth rates of S1-7 and S3-2 were faster than that of wild-type p19 EC cells (Fig. 3A). In wild-type p19 EC cells, the growth rate of RA-treated cells was slower than that of untreated cells. In contrast, the growth rates of S1-7 and S3-2 were not affected by RA treatment (Fig. 3A). These results prompted us to investigate the effect of suppressed CypA expression on RA-induced p19 cell neuronal differentiation. We first examined morphological changes during RA-induced neuronal differentiation (14). As shown in Fig. 3B, RA-induced neurite formation in p19 EC cells but not in S1-7 cells suggests that the CypA knockdown cells lose RA-induced differentiation potential. Next, we examined the expression of β-tubulin III and neurofilament as neuron-specific differentiation markers (15–17). We observed β-tubulin III in RA-induced differentiated p19 EC cells and in p19 EC cells transfected with empty vector, but β-tubulin III was undetectable in either of the knockdown cell lines S1-7 or S3-2 (Fig. 3C). Similarly, up-regulated neurofilament (68 kDa) was observed only in the wild-type p19 cells during retinoic acid-induced neuronal differentiation (Fig. 3D). These results demonstrate that CypA is required for RA-induced neuronal differentiation in p19 EC cells.

To examine whether knockdown CypA also affects Me2SO-induced mesodermal differentiation, we performed Western
To determine levels of RAR expression and RARE-binding activity in the stable knockdown cell line S1-7, we performed Western blot analysis and EMSA for RARβ expression and RARE-binding activity, respectively. RA enhanced RARβ expression in the wild-type p19 EC cells but not in the knockdown cell line S1-7 (Fig. 4B). These results demonstrate that RARβ expression is not inducible by RA treatment when the cells lack CypA. As shown in Fig. 4C, RA-treated cell extracts from control cells also increased binding activity (lane 5) compared with those extracts prepared from cells untreated with RA (lanes 2 and 4). In contrast, RA-treated cell extracts from the knockdown cell line S1-7 did not increase binding activity as exhibited in the control cells (lane 7). The specificity of RARE-binding activity was confirmed by competition assay with a cold probe (lane 3).

Rescue of the Knockdown Phenotype Restores RA-induced Neuronal Differentiation—To ensure that the observed knockdown phenotype as described above is the result of silencing CypA, the intended target, we developed a “knockdown-and-rescue” system to restore siRNA effects. We transfected an expression plasmid, pcDNA3-CypA-re, which contains four silent mutations (Fig. 5A) at the siRNA targeting sequence, into the CypA knockdown S1-7 cell line. Because this expressed, mutated CypA message was not recognized and degraded by existing siRNAs in the established cells, its gene product can function as wild-type CypA. Therefore, we expected a reverted phenotype that can function as wild-type p19 EC cells. pNeoRNAi vector-transfected p19 cells were used as a control cell line. As shown in Fig. 5B, elevated expression levels of CypA were detected in pNeoRNAi-transfected p19 cells, indicating the detectable expression of CypA by ectopically transfected pcDNA3-CypA-wt or pcDNA3-CypA-re expression plasmids. In the S1-7 cell line, expression of CypA was reinstated only by the transfected pcDNA3-CypA-re plasmid (Fig. 5B), demonstrating that the rescue vector successfully restored CypA, the target gene product.

To determine whether the revertants could restore RA-induced neuronal differentiation, either pcDNA3-CypA-wt or pcDNA3-CypA-re was transfected into the S1-7 cell line, which was then treated with RA. The neuron-specific differentiation marker, β-tubulin III, was observed with expression of pcDNA3-CypA-wt but not pcDNA3-CypA-re in S1-7 cells (Fig. 5C). To determine whether the rescued revertant affects RARE-mediated gene regulation, either pcDNA3-CypA-wt or pcDNA3-CypA-re was co-transfected with the pRARE-TK-Luc reporter into the S1-7 cell line in the absence or presence of RA. Similar to the control pNeoRNAi-transfected cells, luciferase activity in pcDNA3-CypA-re-transfected S1-7 cells was enhanced by treatment with RA (Fig. 5D). The activity of luciferase was not significantly enhanced by treatment with RA in the pcDNA3-CypA-wt-transfected cells. pTK-Luc was transfected into cells as a control reporter (Fig. 5D). These results demonstrate that, although p19 cells lacking CypA showed a loss of responsiveness to activation of RAR-mediated gene expression through RARE binding, the restored expression of CypA recovered the activity.

**DISCUSSION**

The effects of transient transfection of siRNAs is well documented, but the data are very limited for knockdown cell lines targeting highly expressed housekeeping genes. Thus, we examined the long term effect of CypA knockdown in neo-resistant transfectants. These cell lines showed a stable and persistent inhibition of CypA up to 12 weeks after initial transfection (Fig. 2). CypA expression was limited to a low steady state level by continuous expression of siRNAs in the stable transfectants. These results provide a model for adopt-
The knockdown phenotype of CypA is followed by wt and pcDNA3-CypA-re were transfected into the S1-7 cell line. Anti-CypA antibodies were used to detect the expression of CypA, using expression of FKBP12 as a control. RA-induced neuronal differentiation of the rescue S1-7 cell line. pcDNA3-CypA-wt and pcDNA3-CypA-re were transfected into the S1-7 cell line followed by RA treatment. Neuron-specific class III β-tubulin was used as a differentiation marker, with expression of FKBP12 as a control. D, luciferase assays were carried out. Cells were transfected with 1 μg of pRARE-TK-Luc or pTK-Luc together with 1 μg of pcDNA3-CypA-wt or pcDNA3-CypA-re and then treated with RA. Levels of luciferase activity from vector-transfected cells in the absence of RA (RA−) were designated as 100%. The data are the average of three independent experiments. pTK-Luc was used as a control.

Fig. 5. Rescue of the knockdown phenotype of CypA restores RA-induced differentiation potential. A, a mutated sequence is shown in the rescue plasmid, pcDNA3-CypA-re. Four silent mutations of nucleotide and amino acid (a.a.) sequences are indicated. B, rescue of the knockdown phenotype of CypA. pcDNA3-CypA-wt, pcDNA3-CypA-re, and pcDNA3 were transfected into the S1-7 knockdown cell line. pNeoRNAi vector-transfected p19 cells were used as a control cell line. Anti-CypA antibodies were used to detect the expression of CypA, using expression of FKBP12 as a control. C, RA-induced neuronal differentiation of the rescued S1-7 cell line. pcDNA3-CypA-wt and pcDNA3-CypA-re were transfected into the S1-7 cell line followed by RA treatment. Neuron-specific class III β-tubulin was used as a differentiation marker, with expression of FKBP12 as a control. D, luciferase assays were carried out. Cells were transfected with 1 μg of pRARE-TK-Luc or pTK-Luc together with 1 μg of pcDNA3-CypA-wt or pcDNA3-CypA-re and then treated with RA. Levels of luciferase activity from vector-transfected cells in the absence of RA (RA−) were designated as 100%. The data are the average of three independent experiments. pTK-Luc was used as a control.

In summary, the powerful tool we have developed to stably suppress CypA expression in p19 cells also can be applied to a variety of biological systems. Specifically, the knockdown-and-rescue system has not only revealed the novel function of CypA, which is required for RA-induced neuronal differentiation in p19 EC cells, it has also demonstrated that this system can be applied in a temporal fashion for the regulation of gene expression in a desired manner. This system also opens new avenues for gene therapy. The design of RNA interfering vectors that target disease-derived transcripts with a point mutation, such as mutant RAS or TP53 oncogenes, can be followed by rescue to form a “wild-type protein” that functions as a wild-type regulatory protein in normal cellular function.

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