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Biochemical and Biophysical Research Communications 337 (2005) 75–81
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Mouse disabled 2 interacting protein 2 functions as a transcriptional repressor through direct binding onto its own promoter

Hyung-Wook Kim, Young-Joon Choi, Jung-Ah Kim, Seong-Ho Bae, Kyoung-Rae Kim, Sung-Soo Park *

School of Life Sciences and Biotechnology, Korea University, 5-ka, Anam-dong, Sungbuk-ku, Seoul 136-701, Republic of Korea

Received 28 August 2005
Available online 13 September 2005

Abstract

The mDaIP2 protein is a mouse orthologue of human Nostrin (a regulator of eNos). The absence of eNos activity in RA-treated F9 cell implies that the protein plays somehow different role from Nostrin. In this experiment, this protein has been shown to repress the expression of its own gene, via a feedback mechanism which involves binding to the promoter region. Data from cotransfection, DNAP, mDaIP2-silenced F9 cell, and EMSA analyses clearly support the repression activity and direct binding of the protein to the promoter region. The protein contains N-terminal FCH domain and C-terminal SH3 domain. The SH3 domain is known to interact with the proline-rich domain of mDab2, while even no function has been reported about the FCH domain. Here, we report a novel function of mDaIP2 as a transcriptional repressor and suggest the possible association of the FCH domain with transcriptional regulation.

Keywords: F9 teratocarcinoma cells; mDaIP2; Transcriptional repression; Autogenous regulation

Murine F9 cells, a line of embryonal carcinoma cells derived from teratocarcinomal cells [1], can be provoked to differentiate into endoderm-like cells by exposure to retinoic acid (RA) [2]. This RA-induced differentiation is known to result in substantial changes in gene expression, which include rapid increases in the rate of expression of the mouse disabled 2 (*mDab2*) gene [3].

mDab2 is a member of the disabled (*dab*) gene family, and its transcript is alternatively spliced into either p96 or p67, in a cell type-specific manner [4]. The p96 isoform is dominantly expressed in both macrophage and tumor cells, whereas the p67 isoform was determined to be dominantly produced in RA-treated F9 teratocarcinoma cells [5]. Both of these isoforms harbor two domains in common. One of these is a phosphotyrosine-interacting domain (PID or PTB), and the other is a proline-rich domain (PRD).

We reported in a previous study that mouse disabled 2-interacting protein 2 (mDaIP2) was obtained using a yeast two hybrid system, in an attempt to identify the partner of mDab2. mDaIP2 consists of 506 amino acids, and its calculated molecular weight is 57.7 kDa. This protein contains an N-terminal FCH domain and a C-terminal SH3 domain [6]. The SH3 domain has been shown to interact with the PRD of mDab2, which was earlier identified to perform a transcriptional activation function, whereas any function has not been reported about the FCH domain. In the RA-treated F9 teratocarcinoma cells, the mDaIP2 and mDab2 genes were expressed differentially in an RA-responsive manner, and both of these were determined to have been localized in both the cytoplasm and the nucleus [6]. A homology search of all NCBI sequences revealed that the amino acid sequence of mDaIP2 shares an 82% identity with human NOSTRIN, which has been implicated in the control of the activity, trafficking, and targeting of nitric oxide synthase (eNos) [7]. As eNos was not detected in the RA-treated F9 cells, we suggested that mDaIP2 somehow functioned in a fashion different to NOSTRIN, with

* Corresponding author. Fax: +82 2 929 1864.
E-mail address: sspark@korea.ac.kr (S.-S. Park).

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doi:10.1016/j.bbrc.2005.08.262

regard to the differentiation of F9 cells. In order to discover the possible role of mDaIP2 in the differentiation of F9 cells, we first conducted a study of the transcriptional regulation of the mDaIP2 gene.

In this study, we have cloned the 5′-regulatory region of the mDaIP2 gene, in order to determine the molecular mechanisms underlying the gene expression and regulation. The cloned region exhibited intrinsic promoter activity, but did not manifest RA responsiveness. A biotinylated DNA precipitation assay, in addition to other measures, indicated that the mDaIP2 protein was capable of repressing the gene promoter via DNA–protein interactions. The characterization of the function of the protein and the processes responsible for the transcriptional regulation of the gene provided us with an important clue as to the mechanism underlying RA-induced F9 cell differentiation.

Materials and methods

Cell culture and retinoic acid treatment. F9 cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% (v/v) fetal bovine serum, at 37 °C in an incubator containing 5% CO_2. In order to elicit differentiation into the visceral endodermal cells, the cells were treated with 1×10^{-6} M all-*trans*-retinoic acid for either 4 or 7 days in aggregation culture, on bacterial Petri dishes.

Subcellular fractionation. The F9 cells were washed twice in PBS and then solubilized in suspension buffer (10 mM Hepes, pH 7.9, 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, and 0.1 mM PMSF) for 20 min, after which lysis buffer (10 mM Hepes, pH 7.9, 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, and 0.1% NP-40) was added and incubated for 20 min on ice. After the cell lysates were centrifuged at 6000*g* for 2 min at 4 °C, the nuclear pellet was resuspended in nuclear extraction buffer (20 mM Hepes, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF) and incubated for 30 min on ice, with intermittent mixing. The nuclear extracts were then collected, after another 15 min of centrifugation at 12,000*g*, at 4 °C.

DNA transfection and luciferase assay. For transient transfection, the F9 cells were treated with retinoic acid (RA) for 4 days and then seeded on the day before the transfection. The cells were incubated with a mixture of DNA and Lipofectamine 2000 reagent (Invitrogen) for 24 h. The DNA mixtures harbored both luciferase plasmid constructs (pGL3) as reporters and pRL-TK, which contains *Renilla* luciferase, as an internal control. The luciferase assay conducted in this study was performed in accordance with the manufacturer's instructions (Promega).

Plasmid constructions. The 5-flanking DNA fragment of the mDaIP2 gene was acquired via PCR from a genomic DNA template which had been prepared from F9 cells. The primers were designed on the basis of a mouse genome database. The PCR products of serial deleted sets were ligated to the *Mlu*I and *Bgl*II sites of pGL3-Basic (Promega), in order to prepare the luciferase reporter constructs for the promoter assay. The pEF-mDaIP2 was acquired via the ligation of the full cDNA sequence of mDaIP2 into pEF-HA, a mammalian expression vector, for expression in the F9 cells. In order to express and purify the mDaIP2 protein in its GST-fused form in *Escherichia coli*, we generated pGEX-mDaIP2, pGEX-mDaIP2N, and pGEX-mDaIP2C via the ligation of a PCR-amplified DNA fragment, which included the coding sequence (amino acids 1–506, 1–290, and 290–506, respectively) of mDaIP2 into the pGEX-4T (Amersham Pharmacia Biotech, Sweden) in-frame. The expression of these constructs was verified via Western blotting, using the mDaIP2 antibody.

PCR primers. We obtained mDaIP2 5′ flanking region deletion sets via PCR. The PCR primers were designed on the basis of the mouse genome database. PCRs were performed using the following synthetic oligonucleotides: 5′-GATTATTTTTAGATGCCCGT-3′ (corresponding to nucleotide −2000 to −1981 of mDaIP2 gene), 5′-GAGAAATGGGACAGAAACAG-3′ (corresponding to −1800 to −1781), 5′-GATGGATGGTTCGGCCAGTA-3′ (corresponding to −1600 to −1581), 5′-AGCTGAGATACTGTGAGACC-3′ (corresponding to −1400 to −1381), 5′-GCTCTGCCCATCATTTTGAG-3′ (corresponding to −1200 to −1181), 5′-CTCTGAGTGACTTGGAGCAG-3′ (corresponding to −1000 to −981), 5′-TGAAGTACAGTGTATCATGA-3′ (corresponding to −800 to 781), 5′-AATCTAGACACCCCCTCAC-3′ (corresponding to −600 to −581), 5′-GCCTAGACCCCAAAGGTCAG-3′ (corresponding to −400 to −381), 5′-AGCTGTTCTGTGCTTTTTCA-3′ (corresponding to −200 to −180), and 5′-GTTGAAATGCTGTCTGCTTT-3′, complementary to −1 to −20 in the genomic sequence.

Biotinylated DNA precipitation. Biotinylated mDaIP2 promoter DNA fragments were prepared via PCR. PCR was conducted using the above-listed synthetic oligonucleotides, and biotinylated 5′-GTTGAAATGCTGTCTGCTTT-3′, which is complementary to positions −1 to −20 in the genomic sequence. We conducted the immobilization of biotinylated DNA and the adsorption of cellular proteins to the DNA as follows. Thirty micrograms of streptavidin magnetic beads (Promega) was washed twice with 2× B&W buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, and 2 M NaCl) and then incubated with 100 ng of biotinylated DNA at room temperature for 1 h in 1× B&W buffer. After extensive washing with 2× B&W buffer and binding buffer (5% glycerol, 20 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 0.15% Triton X-100, 100 mM NaCl, and 4 mM $MgCl_2$), the samples were subjected to 1 h of blocking using binding buffer which contained 1% BSA and were then incubated with 100 μg of nuclear extracts from the F9 cells in 3× binding buffer for 1 h at 4 °C. After five washings with 1× binding buffer, the DNA-bound proteins were subjected to SDS–PAGE, followed by Western blotting, using antibodies.

mDaIP2 silenced F9 cell line construction. Potential target sequences for mDaIP2 were selected in accordance with the manufacturer's guidelines, using the siRNA target finder (Ambion). A series of blast searches verified that the sequences were specific for mDaIP2. Four suitable test sequences were then selected and cloned into pSilencerTM 2.1-U6 neo siRNA expression vector (Ambion). The cloned vectors were transfected into F9 cells and then the transfected cells were selected via neomycin treatments (400 μg/ml). The selected cells (Si-mDaIP2) were then subjected to 4 days of RA treatment. Western blotting showed that mDaIP2 could not be detected in the cells which were selected by RA treatment. We used the pSilencerTM 2.1-U6 neo control siRNA vector (Ambion) as a negative control for this experiment. Cells transfected with control vector were also selected with neomycin and were treated with RA for 4 days. mDaIP2 was also induced normally in a culture of control vector-transfected cells (Si-Con).

Western blotting. RA-treated and -untreated F9 cells were washed in cold PBS and lysed with 1% NP-40 in PBS. About 20 μg of protein in each sample was subjected to SDS–PAGE. Undergoing electrophoresis, the protein in the gel was then electro-transferred onto PVDF membranes (Bio-Rad) and subjected to Western blot analyses. In brief, the blots were incubated with 5% skim milk in TBS (50 mM Tris–HCl, pH 7.5, 150 mM NaCl) for 1 h and then incubated overnight with a 1/2000 dilution of primary antibody. The dilution was prepared in TBST (TBS with 0.1% Tween 20) containing 5% BSA. The membranes were then washed in TBST three times, for 5 min each. The secondary antibody (1/2000 dilution) was added and incubated at room temperature for 1 h. The membranes were then washed three times with TBST, soaked in chemiluminescent reagent (Pierce), and exposed to X-ray film (Kodak). The blot was stripped and re-blotted with anti-β-actin (Sigma) as described above, in order to estimate the relative amount of loaded protein.

Electrophoretic mobility shift assay. The DNA fragments synthesized by the PCR were then labeled with Dig-dUTP (Roche) and used as an electrophoretic mobility shift assay (EMSA) probe. The GST-mDaIP2 protein was purified using a Glutathione Excellose Spin Kit (Bioprogen). Binding reactions were conducted using 20 μg of purified mDaIP2 protein and the nuclear extracts from RA-treated F9 cells, in a binding buffer [50 mM Hepes (pH 7.9), 375 mM KCl, 12.5 mM $MgCl_2$, 0.5 mM EDTA, 5 mM DTT, and 15% Ficoll, and 1 μg poly(dI–dC)]. These mixtures were incubated for 10 min on ice and then for an additional 30 min after the addition of the labeled probe. The reaction mixtures were then loaded onto 5% polyacrylamide gels in TBE. For the competition assay, we added

a 100-fold molar excess of unlabeled probe DNA fragments to these binding reactions. The reaction mixtures for the supershift experiment were incubated for 20 min with 1.0 μl of anti-mDaIP2 antibody at room temperature, after the binding reactions.

Results

Promoter activity of the 5′-upstream region of the mDaIP2 gene

As the initial procedure in an attempt to characterize the regulation of mDaIP2 gene expression occurring during the differentiation of F9 cells, we acquired the 5′-regulatory region (up to −2000) of the gene using PCR. In order to discern the features of the 5′-regulatory region which exhibited promoter activity, F9 cells treated with or without retinoic acid (RA) were transiently co-transfected with pGL3-Basic derived test plasmid (a series of deletions of the 2.0-kb mDaIP2 5′-regulatory regions, fused upstream of the luciferase reporter gene in the pGL3-Basic plasmid) and pRL-TK control plasmid (Fig. 1). Promoter activities were then evaluated via the analysis of *Photinus* luciferase activity in the transfected cell extracts. Luciferase activity was normalized using the *Renilla* luciferase expression from the pRL-TK control plasmid in the same cell extracts. Luciferase activity in each of the constructs is shown relative to the activity from pGL3+, which was used as a positive control plasmid (100%). As shown in Fig. 1, we did not observe promoter activity until the deletion reached the −1200 position. This finding suggests that the repressive *cis*-element might exist in the region between −2000 and −1200. In addition, the promoter region (−1200 to 0) exhibited no significant RA responsiveness, and promoter activities were manifested at consistently lower levels in the RA^+ cells than in the RA^- cells (Fig. 1). This clearly indicated that the retinoic acid response element (RARE) of the mDaIP2 gene did not exist in the region between positions −2000 and 0, and might lie elsewhere.

Biotinylated DNA precipitation assay shows that mDaIP2 protein could bind to 5′-regulatory region of mDaIP2 gene

In order to search for factors which may modulate the promoter activity of mDaIP2, we conducted biotinylated DNA precipitation trials, using the biotinylated mDaIP2 promoter region (−1200 to 0) and the nuclear extracts from F9 cells, either treated or untreated with RA. The specifically bound proteins were eluted and subjected to SDS–PAGE, followed by Western blotting with a variety of antibodies, including c-Myc, Sp1, Smads, RAR, RXR, mDaIP2, etc. In an unexpected finding, mDaIP2 itself could be detected in the nuclear extracts from the RA-treated F9 cells (Fig. 2). Assays using the biotinylated mDab2 promoter and the unbiotinylated mDaIP2 promoter failed to show the detection and therefore we con-

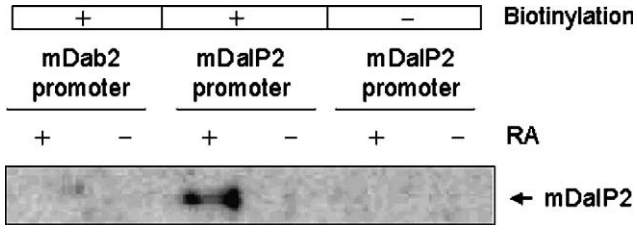

Fig. 2. Biotinylated DNA precipitation assay. F9 cells, treated with (+) or without (−) RA, were fractionated as was indicated in the Materials and methods. Nuclear proteins from the cell lysates bound to the biotinylated or unbiotinylated probe DNA were detected via SDS–PAGE, followed by Western blotting using mDaIP2 antibody. Biotinylated mDab2 promoter and unbiotinylated mDaIP2 promoter DNA were used as the controls.

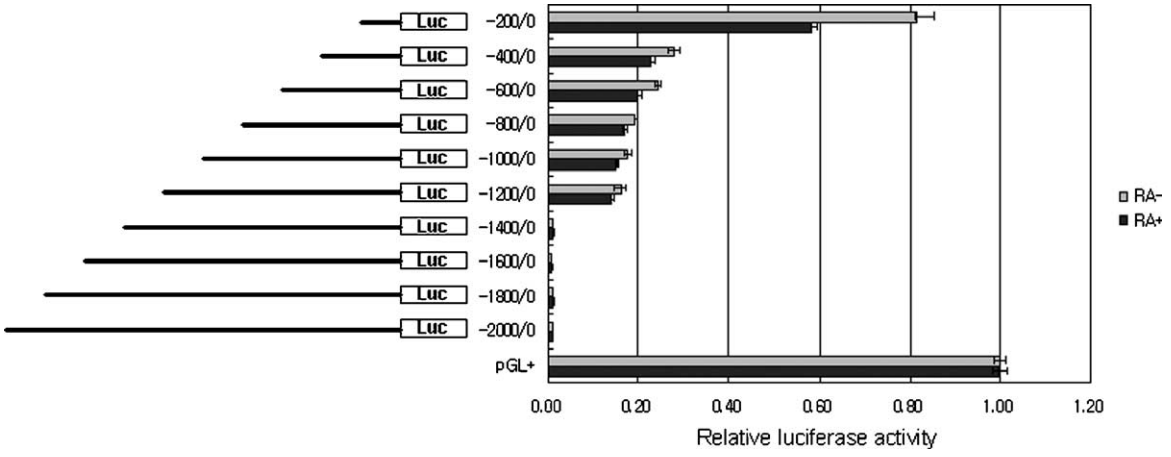

Fig. 1. Promoter luciferase assay with the 5′-regulatory region of mDaIP2 gene. pGL3 Basic vector containing the serially deleted 5′-regulatory region of the mDaIP2 gene or pGL3+ vector were used in the assay. Constructs were transiently transfected into F9 cells which had been treated with RA (+) for 4 days or untreated (−). The 5′-deletion constructs are shown schematically on the left (the horizontal bars indicate the DNA fragments from the 5′-flanking region of mDaIP2 gene, and the boxes marked as 'Luc' code *Photinus* luciferase). We also measured promoter activity from the reporter plasmid. The data are expressed as means ± SE from three independent experiments. Data from the experiments using F9 cells treated with or without RA are indicated by black and gray boxes, respectively.

cluded that this resulted from specific binding of the mDaIP2 protein. When we ascertained that DNA precipitation with the other deleted fragments (−800 to 0, −600 to 0, −400 to 0, and −200 to 0) yielded identical results (data not shown), we surmised that the mDaIP2 protein was able to bind to the 5′-regulatory region of the mDaIP2 gene (−200 to 0).

mDaIP2 protein represses its promoter activity in a dose-dependent manner

In order to characterize the contributions of the mDaIP2 protein to the mDaIP2 gene transcription associated with the results of our biotinylated DNA precipitation assays, we conducted a series of transient transfections. RA-untreated F9 cells were co-transfected with pEF-mDaIP2, and with the luciferase reporter constructs of serially deleted sections of the 5′-regulatory regions (−800 to 0, −600 to 0, −400 to 0, and −200 to 0). Our luciferase assays indicated that the transient expression of mDaIP2 might result in a repression of its promoter activity (Fig. 3A). In order to determine whether or not mDaIP2 was able to influence the activity of the mDaIP2 promoter in a dose-dependent manner, we conducted co-transfections with pEF-mDaIP2 (0–1.5 μg) and pGL3 Basic vector, harboring the promoter region (−200 to 0). Luciferase assays revealed that mDaIP2 could, indeed, selectively repress the activity of the mDaIP2 promoter, and that this took place in a dose-dependent manner (Fig. 3B).

siRNA-mediated silencing of mDaIP2 expression in F9 cells derepresses the repression of mDaIP2 promoter

In order to verify the modulatory effects of the mDaIP2 protein against its own gene promoter, we constructed an mDaIP2-silenced F9 cell line (Si-mDaIP2) and a control cell line (Si-Con), as described in the Materials and methods. As shown in Fig. 4A, endogenous mDaIP2 protein could effectively silence Si-mDaIP2 cells which had been treated with RA for 4 days, whereas mDaIP2 was induced normally in the Si-Con cell line. Using these cell lines, we conducted a series of transient transfections. The Si-mDaIP2 cells and Si-Con cells, which had either been treated with RA for 4 days or had been left untreated, were transfected with pGL3 Basic vector harboring the promoter region (−200 to 0), and with pGL+ control vector. As had been anticipated, the promoter activity in the RA-treated Si-mDaIP2 cells was found to be quite similar to that observed in the RA-untreated Si-mDaIP2 cells, whereas the promoter activity in the RA-treated Si-Con cells was determined to be lower than that observed in the RA-untreated Si-Con cells (Fig. 4B). As the luciferase activity of the pGL+ control vector in these cell lines exhibited no remarkable differences with those which had received RA treatment, our results indicated that the derepression of mDaIP2 promoter activity in the RA-treated Si-mDaIP2 cell line was induced by the silencing of mDaIP2 expression.

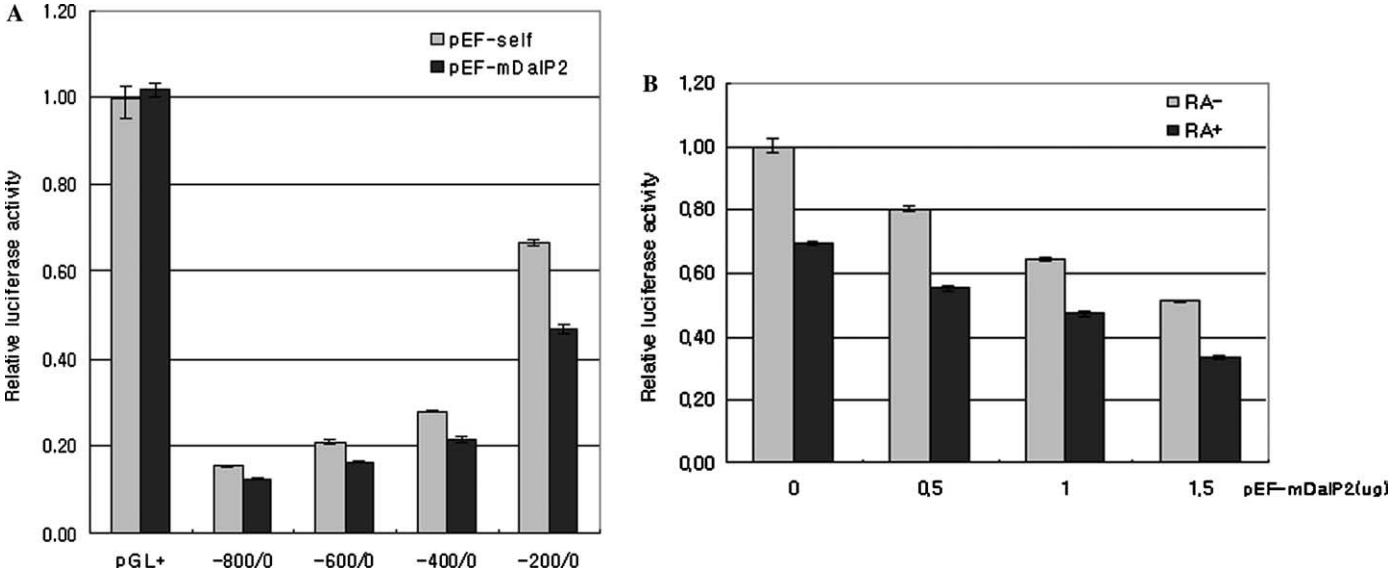

Fig. 3. Co-transfection with mDaIP2 and its promoter regions. (A) pGL3 Basic vector containing the serially deleted 5′-regulatory region of the mDaIP2 gene and pEF-mDaIP2 were used for this assay. The constructs were transiently co-transfected into RA-untreated F9 cells. The luciferase activity from each construct is expressed relative to the activity observed with pGL3+ used as a positive control plasmid. The data from the experiments using pEF-mDaIP2 or pEF self (empty vector) are indicated by black and gray boxes, respectively. (B) pGL3 Basic vector containing the −200 to 0 fragment of the mDaIP2 gene (0.5 μg) and an increasing amount (as indicated) of mDaIP2-expressed vector (pEF-mDaIP2) were co-transfected into F9 cells treated with RA for 4 days (+) or without RA (−). The data from the experiments using F9 cells either treated with or not treated with RA are indicated by black and gray boxes, respectively. The luciferase activity from each construct is shown relative to the activity observed with the RA-untreated F9 cells transfected only with pEF self (empty vector). The data are expressed as means ± SE from three independent experiments.

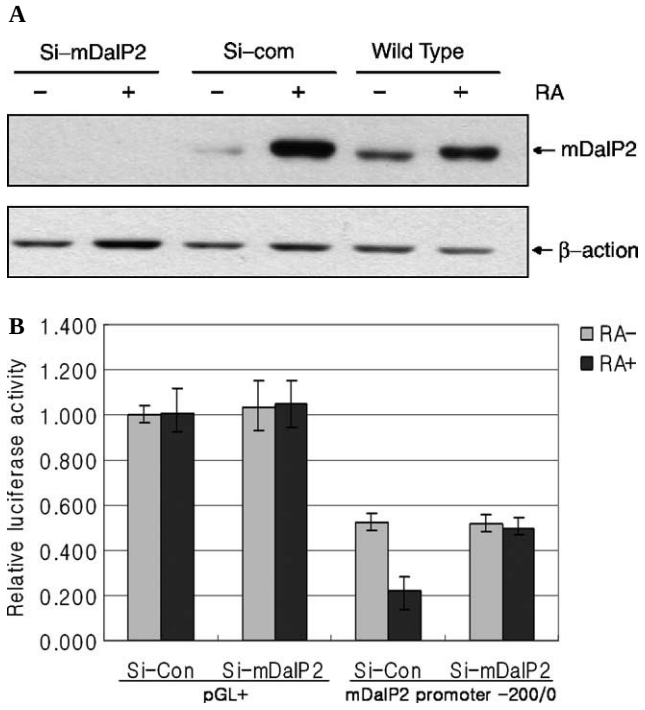

Fig. 4. Transfection into mDaIP2-silenced F9 cells. (A) siRNA-mediated reduction of mDaIP2 expression in the Si-mDaIP2 cells was confirmed via Western blot analysis using mDaIP2 antibody. Equivalent protein loading was verified by β-actin immunoblotting. Si-mDaIP2 cells, Si-Con cells, and wild-type F9 cells were treated for 4 days with RA (+) or untreated (−). (B) Si-mDaIP2 and Si-Con cells treated with RA for 4 days (+) or untreated (−) were transfected with mDaIP2 promoter (−200 to 0) and pGL control. The luciferase activity of each construct is shown relative to the activity from the RA-untreated Si-Con cells transfected with the mDaIP2 promoter (−200 to 0). The data from the experiments using the F9 cells treated with RA or untreated are indicated by black and gray boxes, respectively. The data are expressed as means ± SE from three independent experiments.

Electromobility shift assay shows the direct binding of mDaIP2 protein onto its own promoter

We also conducted EMSA trials, in order to determine whether or not mDaIP2 proteins were able to bind directly to the mDaIP2 promoter. We used the promoter fragment (−200 to 0) as a probe (lanes 1–9, except lane 7) and used the fragment (−2000 to −1800) as a non-specific probe (lane 7). We observed DNA–protein complexes upon the electrophoretic separation of the incubation mixtures of the probe with nuclear extracts from the RA-treated F9 cells (lane 8) and the purified GST-fused mDaIP2 protein (lane 2) (Fig. 5). We did not observe the formation of these complexes as a result of the addition of the unlabeled competitor (lanes 5 and 9), non-specific probe (lane 7), or incubation with partial mDaIP2 (lanes 3 and 4), respectively. Supershift with the mDaIP2 antibody was observed in lane 6 (Fig. 5). These results show that the mDaIP2 protein alone can, indeed, bind to the mDaIP2 gene promoter region, and that this occurs via DNA–protein interactions.

The transcriptional regulation of the mDaIP2 gene appears to be controlled via feedback in the differentiation of F9 cells

As we reported previously, the expression of endogenous mDaIP2 is elevated in F9 cells which are treated with RA for 2–4 days, but the mDaIP2 expression is decidedly attenuated after 4 days of RA treatment [6] (Fig. 6). The attenuation is quite likely to result from both autogenous suppression and relative decrease of the intrinsic promoter activity accompanied with cell differentiation.

In order to evaluate the possibility that the mDaIP2 promoter is regulated in a manner reminiscent of feedback in the RA-induced differentiating F9 cells, we transfected the mDaIP2 promoter reporter construct (−200 to 0) into RA-treated F9 cells (RA 0–7 days). The promoter luciferase assays indicated that the promoter activities of mDaIP2 decrease gradually in accordance with the duration of RA treatment and the increasing mDaIP2 protein expression level (Fig. 6). The continuous down-regulation of the promoter luciferase activities seems to result from the absence of RA-responsive element in the cloned promoter region (−200 to 0) and relative decrease of the intrinsic promoter activity as mentioned above, accompanied with RA-induced cell differentiation, besides the direct mDaIP2 suppressor activity. However, the activities of the pGL control vector were found to exhibit no significant declination. These results imply that the level of mDaIP2 protein

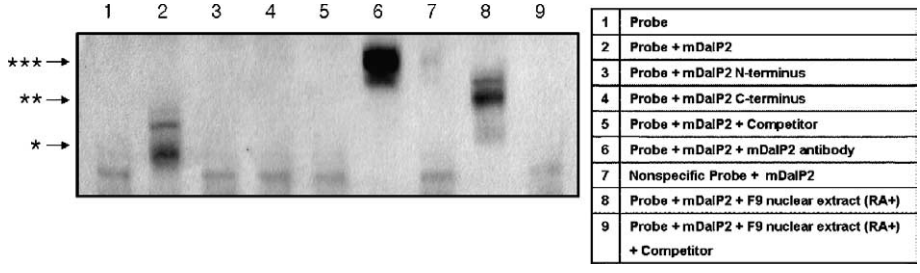

Fig. 5. Electromobility shift assay The Dig-labeled probe was incubated in the absence of protein (lane 1), in the presence of the mDaIP2 protein (lanes 2, 5, 6, and 7), and in the presence of the nuclear extract from F9 cells treated with RA (lanes 8 and 9). The formation of DNA–protein complexes was inhibited in the presence of a 100-fold molar excess of unlabeled competitor (lanes 5 and 9), in the reaction with the partial mDaIP2 protein (N-terminal domain, lane 3; C-terminal domain, lane 4), and in the reaction with the non-specific probe and the mDaIP2 protein (lane 7). The mDaIP2 antibody was added to the reaction mixture in the presence of the mDaIP2 protein to EMSA incubation (lane 6).

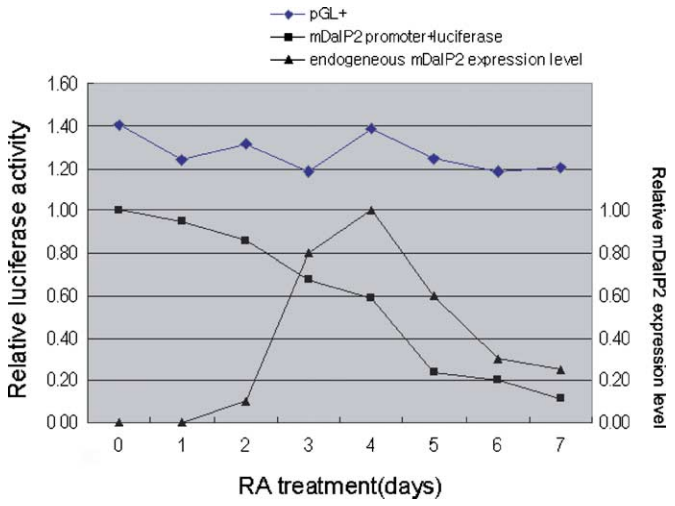

Fig. 6. Promoter activity in differentiating F9 cells. RA-treated F9 cells were transfected with pGL3 Basic vector harboring the −200 to 0 fragment of the mDaIP2 gene and the pGL+ control vector (RA 0–7 day). The luciferase activity from each construct is shown relative to the activity from the RA-untreated F9 cells (RA 0 day) transfected with the mDaIP2 promoter (−200 to 0). The data from the experiments using the pGL+ vector or the mDaIP2 promoter construct are indicated by a diamond (♦) and a square (■), respectively. The data are expressed as means ± SE from three independent experiments. The expression level of endogenous mDaIP2 is shown relative to that of F9 cells treated with RA for 4 days and indicated by a triangle (▲).

expression might be controlled autogenously, via the repression of its promoter activity by this feedback mechanism.

Discussion

The induction of mDaIP2 gene expression has been functionally connected to the process of differentiation in RA-treated F9 teratocarcinoma cells [6]. The amino acid sequences of mDaIP2 have been determined to share 82% identity with human NOSTRIN, which controls the activity, trafficking, and targeting of nitric oxide synthase (eNOS). However, because eNOS was not detected in the RA-treated F9 cells, we proposed that mDaIP2 might function in a somewhat different fashion than NOSTRIN in the process of differentiation in the F9 cells [7].

In our promoter activity assay, the 5′-regulatory region (−2000 to 0) exhibited no promoter activities until deletion reached the −1200 position. This suggests that the silencer element may be located within the deleted region. We also attempted to observe the repressive activity of this region by fusing it to the pGL control vector, but we were unable to observe any repressive activity (data not shown). This repressive mode appears, then, to be specific to the mDaIP2 promoter and to be incompatible with other heterologous promoters (SV40 promoter in pGL+ control vector in luciferase assays). Further investigation will be required in order to clearly determine the precise mechanisms underlying this observed repressive activity. Two other interesting phenomena were observed in the promoter assay. First, the promoter region (−1200 to 0) exhibited no RA responsiveness, although the expression of the mDaIP2 protein occurred in response to RA treatment. The RA-responsive *cis*-element is supposed to lie at elsewhere like the place in far 5′-regulatory region upstream of −2 kb, within the structural gene or in 3′-regulatory region. Second, the variously deleted promoters, from regions −1200 to −200, consistently exhibited lower degrees of activity in the F9 cells which had been treated with RA for 4 days than in the RA-untreated control F9 cells. The lower promoter activity is a contrast to the promoter activity of endogenous mDaIP2 gene, since the endogenous mDaIP2 gene promoter is induced higher in RA-treated F9 cells than in RA-untreated F9 cells. These data drove us to consider the negative feedback effects of endogenous mDaIP2 on the activity of the mDaIP2 gene promoter (−1200 to 0) transfected in RA^+ cells, that is, the autogenous regulation of mDaIP2 gene expression. The autogenous control of mDaIP2 became far more evident once data had been collected regarding the dose-dependent reduction of mDaIP2 promoter activity, when it was co-transfected with mDaIP2-expressing vector. Also, the contrasting restoration of promoter activity in the mDaIP2-silenced F9 cell line further confirmed this result. Also, DNAP and EMSA data allowed us to conclude that the autogenous control of the gene was mediated via the direct binding of mDaIP2 protein to the promoter region.

The FCH domain was first described as a region of homology between Fps/Fes/Fer PTKs and a Cdc42-interacting protein, CIP4 [8]. Amino acid sequence homology searches have detected this FCH domain in many proteins, some of which are implicated in the regulation of cytoskeletal rearrangements [9]. Although the FCH domain of CIP4 was reported to bind to microtubules, the exact role of FCH domain remains largely unknown. In preliminary data, the SH3 domain and FCH domain of mDaIP2 were not involved in DNA binding. Therefore, we suggest that the FCH domain is likely to be implicated in the repressive activity of mDaIP2. The mechanism of DNA binding and transcriptional repression is under investigation.

mDaIP2 shares 82% amino acid sequence identity with human NOSTRIN. In computer structural analyses of these two proteins (http://www.ebi.ac.uk/interpro, http://bmerc-www.bu.edu/psa/request.htm), the proteins were revealed to be functionally identical. How the same protein plays dual roles in two different fashions is a mystery which will, hopefully, be solved in future research.

Acknowledgment

This work was supported by a Korea Research Foundation Grant (KRF-2001-041-DO0185).

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