Down-Regulation of COOH-Terminal Binding Protein Expression in Malignant Melanomas Leads to Induction of MIA Expression

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ABSTRACT

Malignant transformation of melanoma cells to melanoma cells closely parallels activation of MIA expression and involves a promoter region that we referred to previously as a HCR (highly conserved region). The HCR element interacts with the melanoma-associated transcription factor and confers strong activation of the promoter. Furthermore, mutation and deletion studies described in this study revealed that the permissive site for cell-specific promoter activity was located directly 5′ to the HCR region. Changes in the DNA sequence 5′ adjacent to the melanoma-associated transcription factor binding site led to an MIA promoter activity in benign melanocytes and nonmelanocytic cells that usually do not express MIA. Detailed analysis revealed binding of T-cell factor family transcription factors to the repressor element. Because this family is known to interact with COOH-terminal binding protein, we explored the role of COOH-terminal binding protein 1 (CtBP1) in silencing MIA gene expression. By reporter gene analysis, we determined a strict negative regulation of MIA promoter activity in melanoma cells by CtBP1. Furthermore, we observed strong expression of CtBP1 in primary melanocytes but a loss of wild-type CtBP1 expression in malignant melanoma in vitro and in vivo. Therefore, we speculate that CtBP1 has an important negative role in MIA regulation, and loss of CtBP1 is implicated in melanoma progression.

INTRODUCTION

Previously, we described the isolation of MIA, an M, 11,000 protein secreted from malignant melanoma cells into the tissue culture supernatant (1–3). Purified MIA induces detachment of malignant melanocytes and nonmelanocytic cells that usually do not express MIA. Detailed analysis revealed binding of T-cell factor family transcription factors to the repressor element. Because this family is known to interact with COOH-terminal binding protein, we explored the role of COOH-terminal binding protein 1 (CtBP1) in silencing MIA gene expression. By reporter gene analysis, we determined a strict negative regulation of MIA promoter activity in melanoma cells by CtBP1. Furthermore, we observed strong expression of CtBP1 in primary melanocytes but a loss of wild-type CtBP1 expression in malignant melanoma in vitro and in vivo. Therefore, we speculate that CtBP1 has an important negative role in MIA regulation, and loss of CtBP1 is implicated in melanoma progression.

RESULTS

MATERIALS AND METHODS

Cell Lines and Cell Culture Conditions. The melanoma cell lines Mel Im and Mel Ju have been described in detail previously (1, 12). For tissue culture, the cells were maintained in DMEM supplemented with 400 units/ml penicillin, 50 μg/ml streptomycin, 300 μg/ml l-glutamine, and 10% FCS (Sigma, Deisenhofen, Germany) and split 1:3 every third day. Human primary melanocytes derived from normal skin were cultivated in melanocyte medium MGM-3 (Life Technologies, Inc., Eggenstein, Germany) under a humidified atmosphere of 5% CO2 at 37°C. Cells were used in passages 2–4 and not later than 3 days after trypsinization. Cells were detached for subcultivation or assay with 0.05% trypsin, 0.04% EDTA in PBS.

Transfection Experiments. For transient transfections, 3 × 105 cells/well were seeded into 6-well plates and transiently transfected with 0.5 μg of reporter plasmids using the Lipofectamine plus method (Life Technologies, Inc.), according to the manufacturer’s instructions. The following plasmids were transfected: 1386-pGL2 [1386 bp of the human MIA promoter region in pGL2-basic (Ref. 7)], trimer p-GL2 [trimer of oligo c (see Fig. 1A) in pGL2-promoter], mut Y-pGL2 (mutated TCF4 binding site in 1386-pGL2, see Fig. 1A), del Y-pGL2 (deleted TCF4 binding site in 1386-pGL2, see Fig. 1A), pBAT/Bcat [expression vector for β-catenin (Ref. 13)], pCMX-PL1-CtBP1 [expression vector for CtBP1 (Ref. 14)], and pMH TCF4 (expression vector for hTCF4). Twenty-four h after transfection, the cells were lysed, and the luciferase activity in the lysate was measured. To normalize transfection efficiency, 0.2 μg of a pRl-TK plasmid (Promega Corp., Madison, WI) was cotransfected into each well, and Renilla luciferase activity used to normalize efficiency. To ensure equal amounts of transfected DNA in each experiment, the plasmid pRK (Stratagene, Heidelberg, Germany) was cotransfected. All transfections experiments were repeated at least three times.

RNA Isolation and RT-PCR. Expression of TCF4 and CtBP1 mRNA was measured by RT-PCR. For RT-PCR, total cellular RNA was isolated from cultured cells and malignant melanomas using the RNAeasy kit (Qiagen, Hilden, Germany). The integrity of the RNA preparations was controlled on an 1% agarose/formaldehyde gel. First-strand cDNA was synthesized using 1 μg of total cellular RNA as template, 1 μg of random primer (Pharmacia, Freiburg, Germany), 4 μl of 5 × First Strand Buffer (Life Technologies, Inc.), 2 μl of 10 mM DTT, 1 μl of 10 mM deoxynucleotide triphosphates, and 1 μl of SuperScript II (Life Technologies, Inc.) in a volume of 20 μl. For PCR amplification, 2 μl of the cDNA preparation were used as a template, and the following program was applied: 32 cycles of 45 s at 94°C, 30 s at 60°C, and 1 min at 72°C, with a final extension of 5 min at 72°C. The following primers were used: hCBP1for94, CGA CCT CCG ATC ATG AAC; hCBP1rev947, GCC AAA GCT GAA GGG TTC C; TCF4for205, TCA CCA ACA GCG AAT GCC; and TCF4rev809, AGG AAG GAT AGC CTG GCG. PCR products were separated on a 1.8% agarose gel and stained with ethidium bromide. Furthermore, quantitative real-time-PCR was performed on a Lightcycler (Roche, Mannheim, Germany). Two μl of cDNA template, 2.4 μl of 25 mM MgCl2, 0.5 μM forward and reverse primer (hCBP1 LC for, CAT CAT CGT CCG GAT TG; hCBP1 RC rev, CCA TCC GAC AAG TAA GGG), and 2 μl of SybrGreen LightCycler Mix in a total of 20 μl were applied to the following PCR program: 30 s at 95°C (initial denaturation); 20°C/s temperature transition rate up to 95°C for 20 s, 10 s at 58°C, 20 s at 72°C, and 10 s in single
acquisition mode at 87°C, repeated for 40 times (amplification). The PCR reaction was evaluated by melting curve analysis following the manufacturer’s instructions and checking the PCR products on 1.8% agarose gels. To verify amplification of the correct template, PCR products were sequenced.

**Gel Shift Experiments.** The double-stranded oligomeric binding sites oligo c (5’-Ggc tgct agg cat tt tgg ccc atu atu-3’), hum M (5’-GTG AGC TGC TTT GGA CCT TAT C-3’), mut Y (5’-GTG AGC GTG GAC TCA CCT TAT C-3’), and wt-TCP (5’-GAT GTG AAT TGC TTG GTT GGC GTA GC-3’) were phosphorylated and used for gel mobility-shift assays. Nuclear extracts were prepared from primary melanocytes, Mel Im and Mel Ju cells and gel shifts were performed as described previously (7). Competition experiments were performed using a 50-fold excess of the wild-type or mutated binding sites. For supershifting experiments, an antibody directed against TCF4 was used. The antibody was generated in rabbits using GST-TCF4(aa1-52).

**MIA-ELISA.** MIA protein expression was measured using an ELISA system (Roche), following the manufacturer’s instructions. Briefly, monoclonal antibodies coupled with biotin or peroxidase, respectively, were used to quantitate MIA in a 96-well plate coated with streptavidin. ABTS was used as substrate and quantitated at an absorbance of 405 nm.

**Invasion Assay.** Invasion assays were performed in Boyden chambers containing polycarbonate filters with 8-μm pore size (Costar, Badenheim, Germany). Filters were coated with a commercially available reconstituted basement membrane (Matrigel, diluted 1:3 in H2O; Becton Dickinson, Heidelberg, Germany). The lower compartment was filled with fibroblast-conditioned medium as a chemoattractant. Melanoma cells were harvested by trypsinization for 2 min, resuspended in DMEM without FCS at a density 2 × 10^7 cells/ml and placed in the upper compartment of the chamber. After incubation at 37°C for 4 h, filters were removed. Cells adhering to the lower surface were fixed, stained, and counted.

**RESULTS**

Previous studies revealed a 30-bp region in the MIA promoter, which is responsible for melanoma-specific regulation and strong MIA expression (11). We were able to show that a region highly conserved between the human and murine promoter (HCR; Fig. 1A) interacts with the transcription factor MATF and is essential for strong MIA promoter activity in melanoma cells.

In this study, we further investigated the function of the 30-bp region in cell-specific regulation of MIA expression. Therefore, we concentrated on a second conserved binding site located in the 30-bp region 5′ of the HCR region. Deletion of 9 bp or insertion of mutations within this site led to complete loss of melanoma-specific activity of the promoter (Fig. 1B). In reporter gene assays, no activity of the wild-type promoter was found in primary melanocytes or HeLa cells, but strong and specific activity was found in the melanoma cell lines. In contrast, a promoter LUC reporter mutated in the conserved site 5′ adjacent of the HCR region conferred strong activity in HeLa cells and primary melanocytes. From these results, we concluded that the element upstream of the HCR site recruits a negative transcriptional regulator.

Detailed analysis of the repressor element showed that it conforms to a TCF binding site (Fig. 1C). Indeed, gel shift analysis proved binding of TCF4 (Fig. 2A), and TCF4 binding was verified using an anti-TCF4 antibody in supershift experiments (Fig. 2B). Furthermore, competition with a consensus TCF binding site disrupted the gel shift complex (Fig. 2A, Lane 4), and also a mutated form of the region did not form a shifted complex with TCF4 (Fig. 2A, Lane 2). Surprisingly, expression of TCF4 in melanocytes and melanoma cells measured by RT-PCR did not differ in expression level (data not shown), and furthermore, TCF4 binding to the MIA promoter region was equally observed in both melanocytes and melanoma cells (Fig. 2B).

It is known that TCF transcription factors are coactivated by nuclear β-catenin. We therefore performed cotransfection experiments to analyze the effect of β-catenin on MIA promoter activity. Neither a positive nor a negative effect on MIA promoter activity was observed after cotransfecting a series of different amounts of β-catenin expression plasmid (Fig. 3).

More recently, CBP1 was identified to negatively regulate TCF transcription factors. We therefore tested whether CBP1 is involved in regulating the MIA TCF4 element. We analyzed the expression pattern of CBP1 and measured down-regulation of wild-type CBP1 expression in melanoma cells compared with primary melanocytes (Fig. 4A). To rule out cell culture artifacts, further measurements were performed with RNA extracted from malignant melanomas (Fig. 4, A–I) compared with normal skin (Fig. 4B, J–N). To quantify mRNA expression in melanocytes, melanoma cell lines and tissue real-time RT-PCR was performed. The results show clearly the marked down-regulation of CBP1 in melanoma. Amplification of CBP1 on genomic DNA isolated from melanoma cell lines was possible, proving that the gene is not deleted in human melanoma (data not shown). Taken together, our results clearly indicate down-regulation of CBP1 in parallel with up-regulation of MIA in malignant melanoma cells both in vitro and in vivo.

Reporter gene assays were performed to analyze the functional consequences of the loss of CBP1 expression. The activity of the full-length MIA promoter (1386 bp) was strongly repressed after cotransfection of CBP1 in melanoma cells (Fig. 5A). Testing a
CtBP DOWN-REGULATES MIA EXPRESSION

A series of studies have described highly cell type-specific expression of MIA in malignant melanomas and even used MIA serum levels as a surrogate marker to detect melanoma metastasis. By investigating transcriptional mechanisms mediating melanoma-specific gene expression, we previously identified a region in the MIA promoter conferring strong and cell type-specific expression of MIA in malignant melanoma cells. A binding site (HCR) was characterized in this region interacting with a factor referred to as MATF, which strongly activates the MIA promoter in melanoma cells (11).

With our tests of small deletions and mutated constructs of the MIA promoter, we here clearly provide additional evidence that a negative cis-regulatory element is located 5′ adjacent to the HCR region and is critically important in melanoma-specific expression. Defects in this promoter element lead to loss of its specific expression and to unscheduled activity of the promoter in MIA-negative cells, such as HeLa cells or primary melanocytes. Our analysis reveals that TCF4 binds to this region. Interestingly, β-catenin does not seem to function as a corepressor of this TCF-dependent element. We therefore concentrated on the TCF corepressor CtBP1. CtBP1 was first identified as a cytoplasmic protein binding to the COOH-terminal region of the adenoviral protein E1A and attenuating its ability to activate transcription. CtBP recognizes PXDLS motifs in DNA-binding proteins and functions as a transcriptional corepressor in Drosophila, Xenopus, and mammals. The precise mechanisms by which CtBP influences transcription are still under investigation (15). Meanwhile, a number of studies have provided unequivocal evidence that CtBP1 binds to and regulates HMG-box proteins including Sox6 and TCF4 (16, 17). As shown previously for Sox6, this study implicates that TCF4 can act as a transcriptional repressor, depending on the context of coregulatory factors. In vitro reporter gene assays revealed that CtBP1 functions as a strong repressor of MIA promoter activity, and that this repressor function requires the TCF binding element in the MIA promoter. Our expression studies further indicate that loss of TCF/ CtBP1 binding, and consequently loss of suppression of MIA expression in nonmelanoma cells may be important for melanoma progression. In the context of the MIA promoter, TCF is obviously used as a negative regulator in combination with CtBP1. This could be proven by simultaneous transfections of TCF4 and CtBP1 expression plasmids into melanoma cells. Here TCF4 negatively regulates CtBP1-induced repression of MIA promoter activity by interacting with CtBP1.

Further analysis revealed CtBP1 to be strongly expressed in primary melanocytes. In contrast, melanoma cells in vitro and in vivo were shown to have lost or strongly down-regulated wild-type CtBP1 expression. The mechanisms of loss of CtBP1 have to be evaluated in further ongoing studies. As for other proteins, e.g., p16, several mechanisms are feasible, such as mutations, promoter hypermethylation, promoter inactivation, or posttranscriptional mechanisms.

DISCUSSION

A series of studies have described highly cell type-specific expression of MIA in malignant melanomas and even used MIA serum levels as a surrogate marker to detect melanoma metastasis. By investigating transcriptional mechanisms mediating melanoma-specific gene expression, we previously identified a region in the MIA promoter conferring strong and cell type-specific expression of MIA in malignant melanoma cells. A binding site (HCR) was characterized in this region interacting with a factor referred to as MATF, which strongly activates the MIA promoter in melanoma cells (11).

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CtBP has been speculated to be involved in normal cell growth control. It was shown previously that the binding of CtBP to adenoviral E1A correlates with inhibition of E1A plus H-ras cotransformation, tumorigenesis, and metastasis (18–20). Furthermore, a repressor function of CtBP on E2F-mediated transcription via RB was detected (21). In our study, invasion assays pointed to a role of CtBP1 also in cell migration because reexpression of CtBP1 in melanoma cells induced a reduction of the invasive potential. It therefore appears that

Fig. 5. Role of CtBP1 in MIA promoter activity. CtBP1 expression plasmids were cotransfected in several doses (0.5, 0.25, and 0.125 μg/transfection) with MIA promoter reporter plasmids. Either the full-length MIA promoter (1386-pGL2; A) or a pGL2-promoter construct carrying a trimer of oligo c region (trimer c; B) was used. Experiments were performed in the melanoma cell lines Mel Im and Mel Ju. CtBP1 repressed the MIA promoter activity in a dose-dependent fashion. Using the mutated MIA promoter mut Y, no regulation by CtBP1 (0.5 μg/transfection) was found (C). Repression of the MIA promoter activity by CtBP1 was unaffected by cotransfection of β-catenin (D). Bars, SD.
loss of the CtBP1 corepressor function may be a critical event in the pathogenesis of many different malignant tumors.

In summary, we here describe for the first time an important role of CtBP1 in MIA regulation and MIA-dependent effects of melanoma progression. Additional experiments will address the question of whether CtBP1 is not only involved in up-regulation of MIA expression but also of other genes in malignant melanomas.

ACKNOWLEDGMENTS

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REFERENCES


Announcements

MEETING OF THE RADIATION RESEARCH SOCIETY

The annual meeting of the Radiation Research Society will be held at the State University of Iowa, Iowa City, on June 22–24, 1953. The Society will be the guest of the University, and all meetings will be held on the campus. The program will consist of: (1) Two symposia, one on "The Effects of Radiation on Aqueous Solutions," which includes the following speakers: E. S. G. Barron, Edwin J. Hart, Warren Garrison, J. L. Magee, and A. O. Allen. The second is "Physical Measurements for Radiobiology" and companion talks by Ugo Fano, Burton J. Moyer, G. Failla, L. D. Marinelli, and Payne S. Harris. (2) On Monday night, June 22, a lecture by Dr. L. W. Alvarez on meson physics has been tentatively scheduled. On Tuesday night, June 23, Dr. L. H. Gray of the Hammersmith Hospital, London, will speak on a topic to be announced. Dr. Gray's lecture is sponsored by the Iowa Branch of the American Cancer Society. Those desiring to report original research in radiation effects, or interested in attending or desiring additional information, please contact the Secretary of the Society, Dr. A. Edelmann, Biology Department, Brookhaven National Laboratory, Upton, L.I., New York.

ERRATUM

The following correction should be made in the article by Beck and Valentine, "The Aerobic Carbohydrate Metabolism of Leukocytes in Health and Leukemia. I. Glycolysis and Respiration," November, 1952, page 891; substitute for the last paragraph:

The data in Table 8 permit several interesting calculations. If one compares the amount of glucose actually disappearing with the sum of the amount equivalent to lactic acid produced plus that equivalent to O₂ consumption, it is seen that the amount of glucose "cleavage products" exceeds the amount of glucose utilized by 12 per cent in N and 27 per cent in CML and is exceeded by the glucose utilized by 16 per cent in CLL. If the assumption is made that, in this respect, the myeloid and lymphoid cells of leukemia are similar to those of normal blood, it may be that the computed normal figure represents a summation of the myeloid (M) and lymphoid (L) cells that make up the normal leukocyte population. Thus, if M = +0.27 and L = −0.16 and the normal differential is 65 per cent M and 35 per cent L, then

\[ 0.65(0.27) + 0.35(-0.16) = 0.12 \]

a figure identical to the observed +0.12 for normal leukocytes.
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