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Erk 1/2 differentially regulates the expression from the 1G/2G single nucleotide polymorphism in the MMP-1 promoter in melanoma cells

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Abstract

Matrix metalloproteinase-1 (MMP-1) breaks down interstitial collagens, a major component of stromal tissue and a barrier for invading tumor cells. The degradation of collagen by MMP-1 may, therefore, provide one mechanism for facilitating tumor invasion and metastasis. Because of the potential for excessive matrix degradation, the expression of MMP-1 is tightly regulated, often by the mitogen-activated protein kinase (MAPK) pathway. The MAPK signal cascade consists of three separate pathways, the extracellular response kinase (ERK), p38 and Jun N-terminal kinase, which target proteins of the AP-1 and ETS families transcription of the gene. The MMP-1 promoter contains a single nucleotide polymorphism (SNP) at −1607 bp, which creates an ETS binding site by the addition of a guanine (5′-GGAT-3′ or '2G SNP') compared to the 1G SNP (5′-GAT-3′), and enhances MMP-1 transcription. A2058 melanoma cells represent one tumor cell line that is homozygous for the 2G allele and that produces constitutively high levels of MMP-1. Thus, we used these cells to define the mechanism(s) responsible for this high level of expression. We show that inhibition of ERK 1/2 leads to the repression of MMP-1 transcription, and that both the 2G polymorphism and the adjacent AP-1 site at −1602 bp are necessary for high levels of MMP-1 transcription and for the inhibition of MMP-1 expression by PD098059, a specific ERK inhibitor. Furthermore, restoration of MMP-1 levels after ERK 1/2 inhibition requires de novo protein synthesis of a factor necessary for MMP-1 expression. Thus, this study suggests that the ERK 1/2 pathway targets the 2G polymorphism, and that the continuous synthesis of a protein(s) is necessary for the constitutive expression of MMP-1. © 2002 Published by Elsevier Science B.V.

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1. Introduction

The matrix metalloproteinase (MMP) family consists of at least 23 zinc-dependent proteases that together degrade the various components of the extracellular matrix (ECM) [1]. The MMP family is further subdivided into four main groups: the interstitial collagenases, gelatinases, stromelysins and membrane-bound type. One member of the interstitial collagenases is matrix metalloproteinase-1 (MMP-1), which can degrade type I, II, and III col-

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lagens [1]. In most normal cells, physiologic levels of MMP-1 are low, but in pathologic states, such as tumor invasion and metastasis, there may be dysregulation of MMP-1, leading to constitutively high expression [2–5]. For tumor cells to metastasize they must degrade the type IV collagen in basement membrane, which is cleaved by MMP-2 and MMP-9. Once past the basement membrane, however, tumor cells need to invade through the interstitial stroma (collagens I, III) to reach the vasculature and disseminate to distant sites. High constitutive expression of MMP-1 can facilitate this invasive step, thus increasing the metastatic potential of tumor cells. Indeed, the prognosis of patients with tumors expressing high levels of MMP-1 is poorer than those expressing low levels (reviewed in [3]), suggesting that tumors producing high levels of this enzyme have a more aggressive, metastatic phenotype.

MMP-1 can be induced by many physiological stimuli, including cytokines, growth factors and phorbol esters [5,6]. The mechanisms controlling the transcriptional induction of MMP-1 have been studied in detail [5,7,8]. Induction of MMP-1 transcription during wound healing [9] or in rheumatoid arthritis [5] is largely mediated by inflammatory cytokines (e.g. IL-1, TNF), whereas in development, stromal tissue formation is largely mediated by growth factors, such as bFGF [7] and EGF [10]. For both cytokines and growth factors, induction of MMP-1 occurs through activation of multiple signaling pathways. One important component of these pathways is the mitogen-activated protein kinases (MAPK), and a growing body of evidence supports the role of the MAPK pathways in the transcriptional regulation of MMP-1 [5,11,12]. The MAPK consists of three pathways: the extracellular response kinase (ERK), p38 and Jun N-terminal kinase (JNK). The p38 and JNK pathways are activated in response to stress [13], while the ERK pathway is activated by growth factors, usually through Ras, a commonly mutated oncoprotein in cancer [14]. The MAPK pathways target the AP-1 and ETS/PEA3 transcription factor families, which are important regulators of many MMPs [11,12, 15,16]. Most MMPs contain an AP-1 site at approximately −70 bp and this site is important in both basal and induced transcription [5,6,16–18]. In addition, an ETS site is located upstream at −89 bp, and these AP-1 and ETS sites cooperate to regulate transcription [19]. Mutation of this AP-1 site in the MMP-1 promoter reduces basal expression and prevents induction by bFGF [19], IL-1 [5] and phorbol esters [16]. Similarly, AP-1/ETS sites in the stromelysin promoter must act in concert to induce transcription [16], suggesting that AP-1 and adjacent ETS site are crucial in the regulation of MMPs.

One potential target of these signaling pathways in the MMP-1 promoter is a single nucleotide polymorphism (SNP) at −1607 bp, in which the DNA sequence is either 5′-GAA-3′ (1G) or 5′-GGAA-3′ (2G) [20]. The 2G DNA contains the consensus binding site for the ETS family of transcription factors, and is adjacent to an AP-1 consensus sequence at −1602 bp. We have found that there is increased transcription from the 2G SNP compared to the 1G [20], and that tumors with the 2G allele produce higher levels of MMP-1 [20,21]. In addition, the 2G allele has been linked to the progression of malignant melanoma [22], to the incidence of ovarian [23] and endometrial cancers [21], and to the invasiveness of colorectal cancer [24]. In contrast to normal cells that produce low basal levels of MMP-1, some tumor cells, such as the A2058 melanoma cells, have high levels of constitutive expression [20,25,26]. A2058 melanoma cells are an aggressive line established from a brain metastasis and are homozygous for the 2G allele [25]. However, little is known about the factors controlling the high level of expression of MMP-1 in these cells.

In this paper, we studied the signaling pathways that are involved in the expression of MMP-1 in the A2058 melanoma cells, and we examined *cis*-acting sequences in the promoter through which these pathways may mediate their effects. Our results indicate that inhibiting the ERK 1/2 MAPK pathway, which targets the upstream AP-1 and ETS sites in the 2G promoter, reduces the levels of MMP-1. They also suggest that the Ras/Mek/Erk pathway is involved in the continuous production of a protein(s) necessary for this high expression of MMP-1.

2. Materials and methods

2.1. Cell culture

Stock cultures of A2058 melanoma cells were

grown in 150 mm culture plates in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS, L-glutamine, and penicillin/streptomycin (37°C in 5% CO_2), and passaged when they reach confluence [20]. For most experiments, confluent cultures of cells were washed two times with Hanks' balanced salt solution (HBSS) to remove traces of serum and cultured in serum-free DMEM with 0.2% actalbumin hydrolysate (LH) (DMEM/LH). Cells were treated with cycloheximide (Sigma) at 10 μg/ml, varying concentrations of PD098059 and U0126 (Calbiochem), inhibitors of the ERK 1/2 pathway or SB203580 (Calbiochem), an inhibitor of the p38 pathway. All compounds were solubilized in DMSO.

2.2. Northern blot analysis

Total RNA (10 μg) was harvested from cells with Trizol (Gibco) and separated on a 5% formaldehyde gel, transferred to a GeneScreen membrane (NEN Life Sciences) and probed with α-^{32}P-labeled cDNAs for MMP-1, -2, -9 [26] and MT1-MMP [16]. Levels of GAPDH mRNA or 28S rRNA controlled for loading.

2.3. Western blot analysis

Whole cell lysates were harvested by mechanical scraping and collected with 2×SDS buffer [27]. Proteins were separated on 10% Tris–HCl Ready Gels (Bio-Rad) and transferred to Immobilon-P PVDF membranes (Millipore). All membranes were blocked in 5% dry milk (Carnation) at room temperature for 1 h. Blots were probed with antibodies against phospho-p42/44 (pp42/44) and total p42/44 (Cell Signaling) overnight at 4°C, as suggested by the manufacturer. Proteins were visualized by enhanced chemiluminescence (ECL).

2.4. Transient transfection and luciferase assay

One microgram of DNA from promoter constructs from MMP-1 (4372 bp) containing either 1G or 2Gs at −1607 bp, linked to luciferase reporter [20], was transiently transfected into cells using Geneporter (Gene Therapy Systems). Following recovery, cells were washed and incubated with serum-free medium, with or without PD98059, for 24 h, and cell lysates were then assayed for luciferase activity in a luminometer. Mutational and deletional analyses were used to identify target sequences of the signal/transduction inhibitors. All transfections were carried out in triplicate; cells were co-transfected with 25 ng of a CMV-Renilla luciferase construct (Promega) to control for transfection efficiency and data were normalized to this constant.

2.5. PCR-based site-directed mutagenesis

Mutation of the 2G MMP-1 construct containing the mutant AP-1 site at −1602 bp has been previously described [20]; the sequence accession number is AF023338. To construct the 1G MMP-1 promoter with a mutated AP-1 site at −1602 bp, the wild-type site was changed from 5′-TGACTTA-3′ to the mutant form 5′-GTCATTA-3′. An *Aat*II site is located 5′ to the site and an *Eco*RV site is located 3′, which allowed for digestion of flanking sequences and subsequent ligation into a previously digested MMP-1 pGL3 construct with ends compatible with the digested fragment. The *Aat*II sense primer was 5′-CAGTGTATGAGACTCTTCC-3′ and the *Eco*RV antisense primer was 5′-CAGTGGAGAAACACTGGC-3′. The two primers used to make the mutated site were sense 5′-TTAGAAAGATAGTCATTATCTCAAAT-3′, and antisense 5′-ATTTGAGATAATGACTATCTTTCTAA-3′. The sense and the *Eco*RV primers were used to generate a mutant product of 202 bp, and the antisense and the *Aat*II primers were used to generate a mutant fragment of 405 bp. The products of these reactions were combined and used as the template for the *Aat*II and the *Eco*RV primers to create the final fragment containing the mutant AP-1 site. The 455 bp product was excised, purified, and analyzed by agarose gel electrophoresis. Mutagenesis was confirmed by DNA sequencing.

3. Results

3.1. Constitutive MMP-1 expression is ERK 1/2 dependent

The MEK/ERK pathway is often dysregulated in tumors [28] and we hypothesized that this dysregulation may affect the expression of MMP-1. To deter-

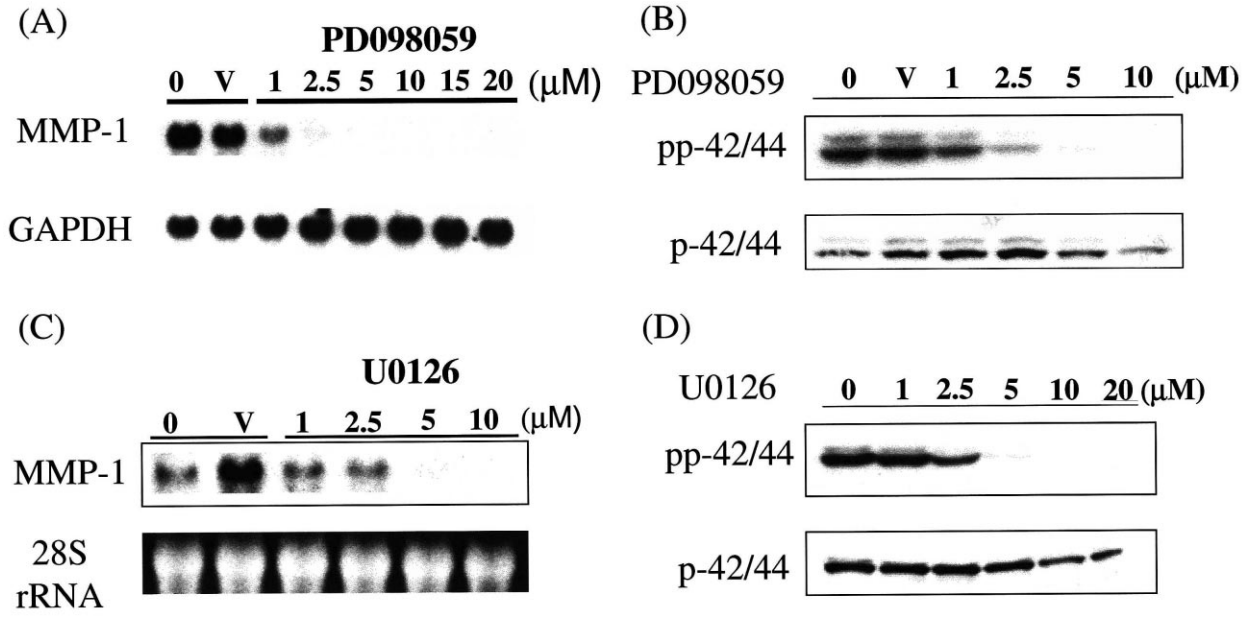

Fig. 1. Northern blot analysis of MMP-1 and Erk phosphorylation state in the presence of increasing concentrations of PD098059 and U0126. (A) Dose response of PD098059 on MMP-1 mRNA levels. A2058 cells were grown until confluent, washed with HBSS and then placed in serum-free medium (0), serum-free medium with 0.05% DMSO (V) or serum-free medium with increasing concentrations of PD098059. After incubating for 24 h, total RNA was isolated, separated on an RNA gel, and probed as described in Section 2. (B) Phosphorylation state of ERK (p42/p44) in the presence of increasing concentrations of PD098059. Cultures were treated in the same manner mentioned above. At 24 h medium was removed and whole cell lysates were harvested in 200 μl of 2×SDS buffer. Whole cell extracts were separated on 10% SDS–PAGE gels, transferred to PVDF and probed for pp42/44 and total p42/44. (C) Dose response of U0126 on MMP-1 mRNA levels. (D) Phosphorylation state of ERK (p42/44) in the presence of increasing concentrations of U0126. Methods are the same as for C. In D, total ERK 1/2 appears as a single band where two bands would be expected. The appearance of only one band may be attributed to several factors, including minimal separation of p42 and p44 (compare D to B) and lower quantities of the p44 isoform (B).

mine whether the constitutive expression of MMP-1 is dependent on this MAPK pathway, A2058 melanoma cells were treated with increasing concentrations (1–20 μM) of PD98059, a specific inhibitor of MEK 1/2 activation [29]. After 24 h treatment, MMP-1 mRNA was analyzed. Fig. 1A shows that levels of MMP-1 mRNA were inhibited in a dose-dependent manner, with total inhibition at 5 μM. To examine the specificity of inhibition by PD098059, we analyzed other MMPs that are also constitutively expressed by these cells [26]. Expression of MMP-9, a gene important in the destruction of the basement membrane (type IV collagen) that is often regulated by the MAPK pathways [30], was minimally down-regulated when normalized to GAPDH mRNA, although only at concentrations of 10 and 20 μM (data not shown). Two other MMPs, MMP-2 and MT1-MMP, another type IV collagenase and a membrane-bound form of these enzymes, respectively, were unaffected by PD098059 (data not shown). Thus, the inhibition of MMP-1 by 5 μM of PD098059 was specific. To support findings that the ERK pathway is involved, U0126, another inhibitor of the ERK pathway, which non-competitively inhibits MEK function [31], was also used. MMP-1 mRNA levels were inhibited at 5 μM U0126 (Fig. 1C), similar to the effects with PD098059.

Phosphorylation of ERK 1/2 on Thr 202/Tyr 204 results in its activation [32,33]. To determine whether inhibition of MMP-1 by chemical inhibitors of the MEK/ERK pathway correlated with decreased levels of phospho-ERK 1/2 (pp42/44), Western blotting with ERK 1/2 specific antibodies for both the phosphorylated and non-phosphorylated forms was performed. We found that in these melanoma cells, ERK 1/2 is constitutively phosphorylated and that

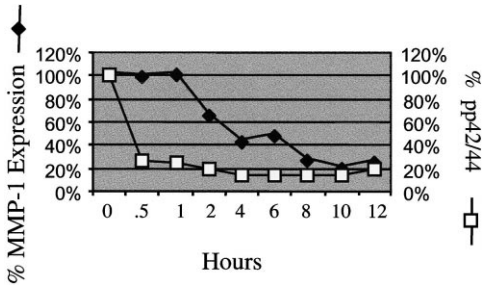

Fig. 2. Inhibition of MMP-1 mRNA by PD098059 lags behind reduction in the phosphorylated form of p42/44. A2058 cells were grown until confluent then transferred to serum-free medium, alone, or with the addition of 5 μM PD098059. RNA and whole cell extracts in 2×SDS buffer were harvested at 0, 0.5, 1, 2, 4, 6, 8, 10, and 12 h. RNA blots were probed with MMP-1 cDNA and protein blots were probed with antibodies to pp42/44 and p42/44. Probed blots were autoradiographed, the developed films were scanned into Adobe Acrobat and band intensities were quantitated by NIH Image. A graphical representation of percent MMP-1 expression and pp42/44 was plotted against time. ♦, MMP-1 mRNA expression; □, pp42/44.

phosphorylation of ERK 1/2 was inhibited completely at 5 μM by both PD098059 (Fig. 1B) and U0126 (Fig. 1D).

Since the MEK/ERK pathway is only one of three possible signaling cascades that comprise the MAP kinase pathways, we wanted to ascertain whether the ERK pathway was working alone or in conjunction with either the p38 or JNK pathway, the stress-activated pathways in the MAPK family. The chemical inhibitor, SB203580, inhibits the p38 pathway at low concentrations (<10 μM) [34] and has been shown to inhibit the JNK pathway at higher concentrations (≥ 10 μM) [34]. In contrast to the inhibitor PD098059, SB203580 had no effect on the expression of MMP-1, even at high concentrations of 20–40 μM (data not shown). The lack of inhibition by SB203580 indicates that the p38 and JNK pathways are not involved in MMP-1 expression. Further, p38 and JNK are not phosphorylated in these cells (data not shown), supporting the finding that these two pathways are not involved in this constitutive expression. Thus, our findings indicate a role for the ERK 1/2 pathway in the constitutive expression of MMP-1 in the A2058 melanoma cells.

As a first step in studying the mechanism of PD098059 inhibition of MMP-1, RNA and whole cell extracts were harvested at different times after addition of 5 μM PD098059 to measure the kinetics of this inhibition. As shown in Fig. 2, reduction of the phosphorylated form of p42/44 (pp42/44) occurs within 30 min of addition of PD098059. This inhibition of pp42/44 precedes inhibition of MMP-1 mRNA steady state by 1.5 h, with inhibition of MMP-1 mRNA beginning approx. 2 h after addition of PD098059. The lag in time suggests that there may be an intermediary protein between the ERK 1/2 pathway and MMP-1 expression, which requires protein turnover before inhibition of MMP-1 occurs. Alternatively, the half-life of the MMP-1 mRNA may be long and even though an inhibition of transcription may have already occurred, a decline may not be seen until later.

3.2. Constitutive expression of MMP-1 requires the de novo production of a protein

Activation of the ERK pathway can lead to the expression of proteins (e.g. transcription factors) de novo and/or to the phosphorylation of existing transcription factors and kinases, thereby activating them [32]. Furthermore, the activation of the ERK pathway may culminate in the expression of MMP-1 through these mechanisms. In order to distinguish between them, phosphorylation and protein synthesis must be separated by inhibiting one function while leaving the other intact. To investigate these possibilities with respect to the constitutive synthesis of MMP-1, cells were pretreated with 5 μM PD098059 or U0126 for 24 h. Inhibitors were then removed and cells were allowed to recover for 12 or 24 h. During the recovery period, selected cultures were treated with cycloheximide to determine whether protein synthesis was required to re-institute high constitutive expression of MMP-1 mRNA. As expected, a 24 h treatment with PD098059 or U0126 completely inhibited the expression of MMP-1 mRNA (Fig. 3A). In cells treated with cycloheximide alone, we found that MMP-1 expression was slightly super-induced, 1.16 and 1.37 times over mRNA levels in control cells in serum-free medium (LH) at 12 and 24 h, respectively, although this increase was not statistically significant ($P=0.114$). A similar finding has been reported for MMP-3 gene expression in cycloheximide-treated cells and it has been suggested that this increase may be due to stabilization of mRNA [35].

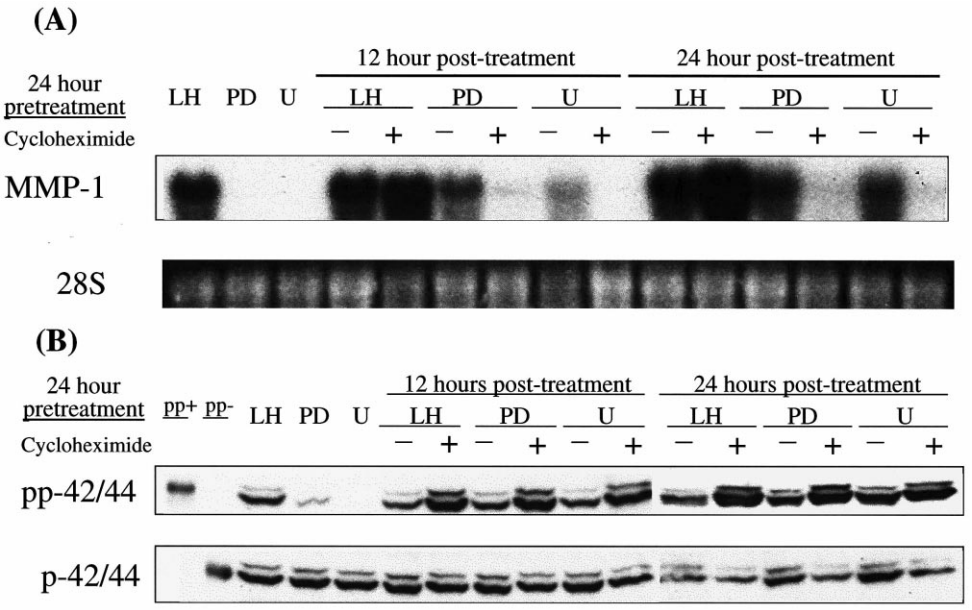

Fig. 3. The effects of cycloheximide on MMP-1 transcription after inhibition with PD098059 and U0126. A2058 melanoma cells were incubated in serum-free medium (LH) with or without 5 μM PD098059 or U0126 for 24 h. Post incubation, selected cultures were transferred to serum-free medium with or without cycloheximide for either 12 or 24 h. (A) Northern blots of RNA were probed with MMP-1 cDNA as described in Section 2. (B) Whole cell extracts were analyzed on a SDS–PAGE gel and probed for pp42/44 and total p42/44 as in Fig. 1. pp+, phosphorylated control; pp−, non-phosphorylated control.

Fig. 3A shows that by 12 or 24 h after removal of PD098059 or U0126, expression of MMP-1 returned to control levels in cells from which the inhibitors had been removed without the subsequent addition of cycloheximide, but was either minimal or absent in the samples treated with cycloheximide. The small amount of mRNA expression in samples that contained cycloheximide can be attributed to incomplete inhibition of translation as determined by ^{3}H-leucine incorporation, which was inhibited 85% (data not shown). These results suggest that the constitutively high expression of MMP-1 requires de novo protein synthesis of a factor(s) that is depleted during the 24 h treatment with PD098059 or U0126.

To support this conclusion and to examine whether the lack of MMP-1 mRNA recovery was possibly due to a decrease in total ERK 1/2 or ERK 1/2 activity, whole cell extracts from the previous experiment were probed for p42/44 and pp42/44. In all samples from cycloheximide-treated cells, there was an increase in the phosphorylated forms of ERK compared to controls even when total levels of ERK declined (Fig. 3B). Increased ERK phosphorylation may be the result of the reduction of a phosphatase due to cycloheximide [36], and the decrease in the level of total ERK was probably a function of decreased protein synthesis. Thus, these findings support the concept that PD098059 depleted a protein(s) necessary for MMP-1 mRNA expression.

3.3. MMP-1 inhibition by PD098059 is transcriptional, and the 1G/2G polymorphism is inhibited through different mechanisms

To determine whether PD098059 is suppressing MMP-1 gene expression by inhibiting transcription, 4.3 kb of promoter DNA containing either the 1G or the 2G allele linked to a luciferase reporter were transiently transfected into A2058 cells, which were then treated with increasing concentrations of PD098059. Fig. 4A shows that transcription of the 2G allele is enhanced compared to the 1G allele, in keeping with our previous studies [20]. Further, transcription of both the 1G and 2G promoter constructs was inhibited in a dose-dependent manner. PD098059 equally repressed expression from the 1G and 2G constructs, with approx. 50% inhibition at 5 μM PD098059 (Fig. 4A), a concentration where

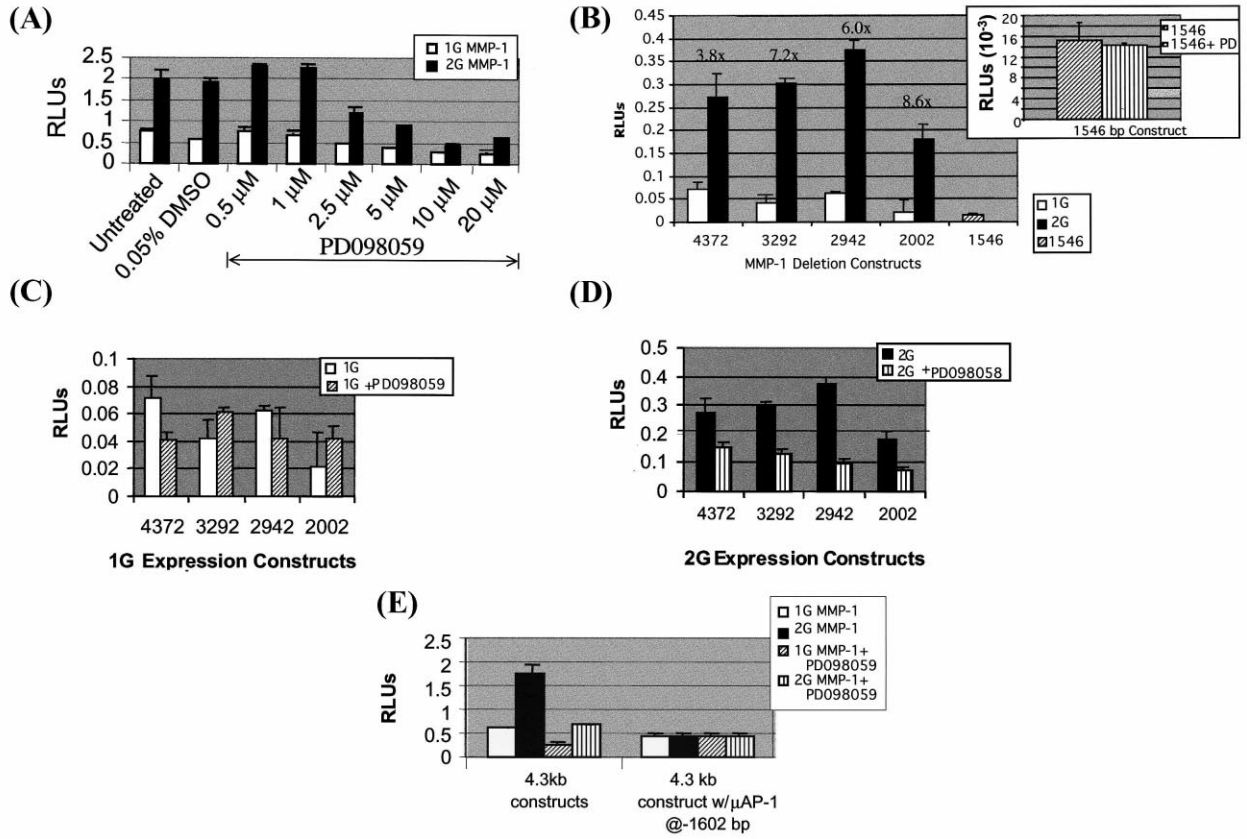

Fig. 4. Effects of PD098059 on the 1G and 2G MMP-1 promoter constructs. (A) Dose–response curve of PD098059. Triplicate cultures of A2058 melanoma cells were transiently transfected with the 4.3 kb promoter constructs containing either 1G or 2G as described in Section 2. Cultures were harvested and analyzed for both reporter activity and transfection efficiency. The fold expression of 2G over 1G is located above the data sets being compared. DMSO (V) at 0.05% was used as the vehicle control. In all experiments, data points were averaged from triplicates in three independent experiments and represent mean ± S.D. B–D represent data from the same experiment. (B) Deletional analysis of 1G/2G promoter constructs. Triplicate cell cultures were transiently transfected with deletion constructs and analyzed. (Inset) Comparison of construct deleted at −1546 bp, either untreated or treated with 10 μM PD098059. Untreated construct is enlarged from B. (C) 2G deletion constructs treated with 10 μM PD098059. (D) Treatment of 1G deletion constructs with 10 μM PD098059. (E) Mutational analysis of the AP-1 site at −1602 bp in 4372 bp MMP-1 promoter in cells with or without 10 μM PD098059. The different levels of RLUs reflect the fact that transfection efficiency can vary from one experiment to another.

the endogenous mRNA was also repressed (Fig. 1A). The slight discrepancy in the effective concentration of PD098059 inhibiting MMP-1 mRNA and luciferase protein may be a function of the differences between these two assays. Since both the 1G and the 2G promoters were inhibited equally, this suggests that the 2G SNP may not be the sole target of the ERK 1/2 pathway.

To identify the DNA targets of ERK 1/2, a series of deletion constructs ranging from −4372 bp to −1564 bp, containing either the 1G or the 2G allele [20], were transiently transfected into cells with or without PD098059. In all constructs, the 2G construct showed higher expression compared to the 1G construct, and deletion of upstream sequences did not diminish the fold increase of the 2G containing promoter over the 1G (Fig. 4B). Rather, deletion of upstream DNA increased the fold expression of the 2G over the 1G. Indeed, in the 4372 bp construct, expression of the 2G DNA was 3.8 times greater than that of the 1G, while in the deletion constructs, expression of the 2G DNA compared to that of the 1G DNA increased 7.2-, 6.0- and 8.6-fold, in order of decreasing length (Fig. 4B). However,

deleting the promoter to −1564 bp, which removes the upstream AP-1 site at −1602 bp and the SNP at −1607 bp, drastically reduced expression, which was not further inhibited by PD098059 (Fig. 4B). All constructs containing 2G SNP were inhibited by PD098059 (Fig. 4C). These findings confirm that the increase in expression of the 2G promoter over the 1G promoter is primarily due to the extra G at −1607 bp. In contrast, inhibition of reporter expression by PD098059 in the 1G deletion constructs was only observed in the 4.3 kb construct. This finding suggests that in the absence of the 2G allele, PD098059 may inhibit MMP-1 through a site upstream from −3292 bp (Fig. 4D) (see Section 4).

Finally, since AP-1 and ETS sites cooperate to drive transcription [18,20,37,38], we used mutational analysis to determine the role of the AP-1 site at −1602 bp. Thus, 4.3 kb constructs containing either 1G or 2Gs, each with a mutated AP-1 site at −1602 bp, were transiently transfected into cells with or without PD098059. The wild-type constructs respond as expected, with an increase in transcription by the 2G DNA over the 1G, and with an inhibition of both (Fig. 4D) alleles with PD098059. However, when the AP-1 site was mutated, there was no difference in the level of transcription between either construct and PD098059 did not inhibit transcription (Fig. 4E). Together, these data suggest that both the ETS site (2Gs) at −1607 bp and the AP-1 site at −1602 bp are needed to attain the enhanced transcription seen in the 2G allele compared to the 1G. In addition, both sites are necessary for the inhibition of the constructs containing the 2G SNP by PD098059.

4. Discussion

In normal physiology, the expression of MMP-1 is generally very low. During situations such as wound healing, embryonic development and certain diseases, expression of MMP-1 can be induced to high levels by multiple stimuli, e.g. growth factors [19] and cytokines [5]. Many inducers of MMP-1 activate the MAPK signaling pathways and these pathways have been shown to regulate MMP-1 [11,12,28,39]. However, some tumor cells, including the A2058 melanoma cells, express high levels of MMP-1 in the absence of external stimulation [20,25,26]. In this paper we show that this high expression is due to a constitutively active MEK/ERK pathway, which can be blocked by two chemical inhibitors of this pathway, PD098059 and U0126. In contrast to other studies, where MMP-1 is induced in chondrocytes and fibroblasts by inflammatory cytokines [5,11], the p38 and JNK pathways are not involved in the expression of MMP-1 in the melanoma cells. Thus, depending upon the cells and the stimuli, the signal/transduction pathways leading to MMP-1 expression differ, emphasizing that MMP-1 gene expression can be controlled by multiple mechanisms.

The MAPK pathways can increase transcription by two mechanisms: by increasing the activity of kinases and transcription factors by phosphorylating them, or by increasing the levels of these proteins themselves [40,41]. When cells were treated for 24 h with the MEK/ERK inhibitors and then given cycloheximide after removal of inhibitors, they were unable to recover full expression of MMP-1. Therefore, we can conclude that the expression of MMP-1 is dependent on the de novo synthesis of a protein(s). As yet, these proteins have not been identified, although potential candidates include members of the AP-1 and ETS families of transcription factors that are expressed by these cells (U. Benbow et al. (2001) submitted).

The AP-1 and ETS families of transcription factors are common targets of the MAPK pathway and are stronger *trans*-activators when they bind to adjacent sites and work in concert [19,42]. There are a number of AP-1 and ETS sites in the MMP-1 promoter [19,37,43], but the 2G polymorphism at −1607 bp produces an additional ETS site that is adjacent to an existing AP-1 site at −1602 bp. Our data suggest that PD098059 may be targeting this site since the expression from the 2G constructs was inhibited throughout the deletion series. Furthermore, in constructs containing the 1G polymorphism, only the −4.3 kb construct was inhibited by PD098059, suggesting that in the absence of the 2Gs at −1607 bp the MEK/ERK pathway targets a sequence upstream from −3292 bp. Potential upstream sites for PD098059 inhibition in the 1G construct are an AP-1 site at −3475 bp, and two ETS binding sites at −3238 bp and −3908 bp. Thus, in the absence of the 2G site at −1607 bp, the *trans*-

activating factors may find alternative *cis*-activating sequences within the MMP-1 promoter through which they activate gene expression. Finally, when sequences upstream from −1564 bp were removed, or the AP-1 site at −1602 bp was mutated, regulation by the MEK/ERK pathway was abolished. Since AP-1 factors tether ETS proteins and increase their binding affinity, AP-1 factors are often necessary for ETS proteins to transactivate transcription and, thus, deletion of the AP-1 site at −1602 bp may have deleterious effects on the transcriptional activity of the 2G SNP.

Since the downstream targets of ERK 1/2 are often transcription factors, it is intriguing to speculate that this protein(s) is a transcription factor(s) whose expression is controlled by the ERK 1/2 pathway, which then regulates MMP-1 transcription. Thus, inhibition of MMP-1 by ERK 1/2 inhibitors would be a secondary event, as suggested in Fig. 2. Furthermore, it will be important to determine whether AP-1 or ETS family members are down-regulated in response to PD098059, and whether these transcription factors contribute to the constitutive expression of MMP-1 by targeting these sites. The possibility that the same factor(s) may be responsible for the expression of both alleles of the promoter but target different sites in each promoter and induce different levels of transcription is an interesting concept. If both alleles are targeted by the same factors, it may be possible to induce or repress MMP-1 expression, whether the genotype is 1G and/or 2G, by targeting the common factor, or the pathways leading to its activity. Ultimately, by understanding the mechanisms that cause high constitutive expression of MMP-1, we may be able to devise treatments that inhibit the pathways regulating MMP-1 production, and thus reduce the metastatic ability of tumors.

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