Loss of Activator Protein-2α Results in Overexpression of Protease-activated Receptor-1 and Correlates with the Malignant Phenotype of Human Melanoma*

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Increasing evidence implicates the protease-activated receptor-1 (PAR-1) as a contributor to tumor invasion and metastasis of human melanoma. Here we demonstrate that the metastatic potential of human melanoma cells correlates with overexpression of PAR-1. We also provide evidence that an inverse correlation exists between the expression of activator protein-2α (AP-2) and the expression of PAR-1 in human melanoma cells. Repression of AP-2 in WM266-4 melanoma cells, which are AP-2-negative, resulted in decreased mRNA and protein expression of PAR-1. The promoter of the PAR-1 gene contains multiple putative consensus elements for the transcription factors AP-2 and specificity protein 1 (Sp1). Chromatin immunoprecipitation analysis of the PAR-1 promoter regions bp −365 to −329 (complex 1) and bp −206 to −180 (complex 2) demonstrated that Sp1 was predominantly bound to the PAR-1 promoter in metastatic cells, whereas AP-2 was bound to the PAR-1 promoter in nonmetastatic cells. At the protein level, AP-2 was colocalized with PAR-1 in metastatic cells. In vitro analysis of complex 1 demonstrated that AP-2 and Sp1 bound to this region in a mutually exclusive manner. Transfection experiments with full-length and progressive deletions of the PAR-1 promoter luciferase constructs demonstrated that metastatic melanoma cells had increased PAR-1 promoter activity compared with low and nonmetastatic melanoma cells. We obtained data indicating exogenous AP-2 expression reduced promoter activity, whereas transient expression of Sp1 further increased expression of the reporter gene. Mutational analysis of complex 1 within PAR-1 luciferase constructs further demonstrated that the regulation of PAR-1 was mediated through interactions with AP-2 and Sp1. Our data suggest that loss of AP-2 in metastatic cells alters the AP-2/Sp1 ratio, resulting in overexpression of PAR-1. Taken together, our results provide strong evidence that loss of AP-2 correlates with overexpression of PAR-1, which in turn contributes to the acquisition of the malignant phenotype of human melanoma.

We have previously reported that loss of the transcription factor activator protein-2α (AP-2) is associated with the transformation of melanoma cells from radial growth phase to vertical growth phase. Our studies demonstrated that nonmetastatic melanoma cell lines expressed high levels of AP-2, whereas highly metastatic melanoma cell lines did not express AP-2 (1–3). Furthermore, we showed that transfection of highly metastatic melanoma cells with full-length AP-2 significantly reduced the tumorigenicity and metastatic potential of these cells in vivo (2, 3). Similarly, inactivation of AP-2 by stable transfection with a dominant negative AP-2 gene (AP-2B) into AP-2-positive primary cutaneous melanoma cells increased tumor growth in vivo (4). These observations have been supported by several studies in human melanoma clinical specimens.

We have previously demonstrated that loss of AP-2 is an important molecular event in melanoma progression, which results in deregulation of AP-2 target genes involved in tumor growth and metastasis. For example, loss of AP-2 expression in metastatic melanoma cells results in overexpression of the melanoma cell adhesion molecule (MCAM/MUC18) (2) and lack of expression of the tyrosine kinase receptor c-KIT (3). Increased expression of MUC18 allowed the metastatic cells to adhere to the endothelial cells in blood vessels and supported their migration to the metastatic site (5), whereas low c-KIT expression rendered the cells resistant to apoptosis (6). In addition, inactivation of AP-2 in AP-2-positive primary cutaneous melanoma cells by means of transfection with a dominant negative AP-2 gene (AP-2B) led to deregulation of the matrix metalloproteinase-2 gene (4). Furthermore, immunohistochemical analyses of advanced primary and metastatic melanoma clinical specimens demonstrated that the loss of AP-2 expression correlated with low p21/WAF1, E-cadherin, and c-KIT expression and poor prognosis (7, 8). Functional AP-2-binding elements have been identified in other genes involved in the progression of human melanoma, including p21/WAF1 (9), intercellular adhesion molecule (10), c-erbB-2/HER-2/neu (11–13), plasminogen activator inhibitor type I (14), insulin-like growth factor-binding protein-5 (15), transforming growth factor-α (16), vascular endothelial growth factor (17, 18), E-cadherin (19), and hepatocyte growth factor (20).

The protease-activated receptor-1 (PAR-1) is a unique G-coupled protein receptor, which belongs to the protease-activated receptor family. Activation of PAR-1 involves proteolytic cleavage of the extracellular amino-terminal domain by thrombin to unmask a new amino terminus capable of serving as a tethered ligand for the receptor, which leads to downstream cell signaling events that evoke a variety of cellular responses (21). Overexpression of PAR-1 has been detected in numerous human cancers, including colon (22), laryngeal (23), breast (24), pancreatic (25), and oral squamous cell carcinomas (26). Recent...
that loss of AP-2 may contribute to PAR-1 up-regulation in TATA and CAAT sequences, and multiple AP-2-binding elements were necessary for PAR-1 promoter both in vivo and in vitro. Analysis of the DNA-binding and protein expression of AP-2 and Sp1 in a panel of melanoma cell lines revealed a marked decrease in the ratio of AP-2 to Sp1 expression, which correlated with an over-expression of PAR-1 in metastatic melanoma cells. These results provide strong evidence for an additional mechanism by which loss of AP-2 expression and overexpression of PAR-1, contributes to the malignant phenotype of human melanoma.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—The human melanoma cell line A375SM (30). However, the mechanism for up-regulation of PAR-1 expression in malignant melanoma is unknown. The regulatory region of the PAR-1 gene has been cloned, and DNA sequence analysis indicates the presence of multiple putative AP-2 and specificity protein 1 (Sp1) regulatory elements at the proximal 3′ region of the promoter (31, 32). The genomic organization of the promoter shares remarkable similarities with other AP-2 target genes, such as a G + C-rich sequence, lack of conventional TATA and CAAT sequences, and multiple AP-2-binding elements. Previously, it has been shown that Sp1 transactivates the PAR-1 promoter in human endothelial cells (33). We hypothesized that PAR-1 expression is regulated by AP-2 and that loss of AP-2 may contribute to PAR-1 up-regulation in malignant melanoma.

Here we provide further evidence that AP-2 is involved in the etiology of malignant human melanoma. We observed a direct correlation between the expression of PAR-1 and the metastatic potential of human melanoma cell lines. Thus, we demonstrated that an inverse correlation exists between the levels of expression of AP-2 and PAR-1 in human melanoma cell lines. Furthermore, we found that enforced expression of AP-2 in the human metastatic melanoma cell line WM266-4, which is AP-2-negative, resulted in decreased expression of PAR-1. In determining the mechanisms of PAR-1 gene regulation, we analyzed specific DNA elements of its promoter. The nuclear proteins AP-2 and Sp1 were found to bind to elements of the PAR-1 promoter both in vivo and in vitro. Analysis of the DNA-binding and protein expression of AP-2 and Sp1 in a panel of melanoma cell lines revealed a marked decrease in the ratio of AP-2 to Sp1 expression, which correlated with an over-expression of PAR-1 in metastatic melanoma cells. These results provide strong evidence for an additional mechanism by which loss of AP-2 expression and overexpression of PAR-1, contributes to the malignant phenotype of human melanoma.
nology, Inc., Lake Placid, NY). The immune complexes were eluted by adding 250 μl of 1% SDS in 0.1 M NaHCO3, to the pelleted beads and then incubated at room temperature for 15 min. Then 20 μl of 5 M NaCl was added, and the complexes were incubated at 65 °C for 4 h. The DNA was recovered by phenol/chloroform extraction and ethanol precipitation using 20 μg of glycerol as a carrier. The precipitated DNA was then dissolved in 20 μl of TE buffer and analyzed by PCR. PAR-1 primer sequences (5'-ACT TCT AGG CCC GGC ACT G-3' and 5'-GCT AAG ATC AGG GTC CAA GC-3') were used. The PCR was subjected to an initial denaturation step (2 min at 96 °C), followed by 30 cycles of denaturation (1 min at 94 °C), annealing (1 min at 60 °C), and extension (1 min at 72 °C). Then reaction was subjected to a final extension time of 5 min at 72 °C. PCR products were analyzed on a 3% agarose gel containing ethidium bromide.

**Semiquantitative Reverse Transcriptase-PCR**—One microgram of total RNA was reverse-primed with an oligo(dT) primer and extended with Maloney murine leukemia virus reverse transcriptase (Clontech, Palo Alto, CA). The PCR was performed, using the Clontech Advantage cDNA PCR kit, in a 50-μl reaction mixture containing 1× PCR buffer, 5 μl of cDNA, 0.2 mM dNTP, and 2.5 units of Taq polymerase. For quantitation of AP-2, cDNA was amplified by PCR using specific primers for human AP-2 (sense, 5'-CTG CCA ATG TTA ACC GGC-3' and antisense, 5'-TAG TTC TGC AGG GCC GTG-3') and the housekeeping gene GAPDH (sense 5'-GAG CCA CAT CGT GCA GAC-3' and antisense, 5'-CTT CTC ATG GTT CAC ACC C-3'). AP-2 and GAPDH cDNAs were amplified by PCR in the same reaction mixture. PCR was subjected to an initial denaturation for 2 min at 94 °C, followed by 27 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, and extension at 72 °C for 1 min, with a final elongation step at 72 °C for 5 min. For PAR-1 quantitation, specific primers (5'-GCA GAG CCC GGG ACA ATG-3' and 5'-AGA TGG CCA GAC AAG TGA-3') were used. The PCR was carried out by an initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 63 °C for 1 min, and extension at 72 °C for 1 min. A final elongation step was carried out at 72 °C for 5 min.

**Expression Constructs—pcDNA3.1 (Invitrogen)** was a mammalian cell expression construct in which expression is driven by the cytomegalo- virus promoter and provides neomycin resistance. The pcDNA3.1 vector was constructed for stable and transient transfections and was created by cloning the AP-2 cDNA into the EcoRI site of pcDNA3.1. The pcDNA3.1-Sp1 construct that contained the cDNA encoding for Sp1 was used for transient transfections.

**Construction of PAR-1 Luciferase Plasmids**—DNA from human melanoma WM266-4 cells was prepared with a Qiagen genomic tip system (Valencia, CA) according to the manufacturer's instructions. The nucleotide upstream of the translation start site was numbered −1. The 1400-, 890-, and 500-bp genomic fragments upstream of the translation start site were generated by PCR from genomic DNA using the Qiagen PCR kit. The underlined restriction sites in each primer were added to the 5′ ends of the primers were used to initiate the 1400-bp product with primers complementary to the regulatory region of the PAR-1 gene (sense 5’-GGT ACC AGT GGC AAA GCA ACT TA-3’ and antisense 5’-CTC TGC CCT CTC GAC TGC GG-3’). The 890-bp product of the PAR-1 promoter was generated with the following primers: sense 5’-GGT ACC CCG TCT TCT TCT CTC CTT GAC TCC TGC GG-3’ and antisense 5’-GGT ACC CTC TCT CCT GAC TGC GG-3’. Similarly, the 500-bp region of the PAR-1 promoter was generated with the primers sense 5’-GGT ACC GGC TCC CAT TAC GAG GAC-3’ and antisense 5’-GGT ACC CTC TCT CCT GAC TGC GG-3’. The PCR products were subcloned in the promoterless pGL3 basic vector (Promega) and digested with XhoI and KpnI, generating the constructs for human AP-2 (sense, 5'-CTG CCA ATG TTA ACC GGC-3' and antisense, 5'-CTG TGC GCC AAC CCC GCC CCC GAA CTG CCT CCC TAC TGG-3'; M3 sense, 5'-CTC GTA GGG CAG TCC GGG GGG CTT GCC GCA CAG A-3'; M3 antisense, 5'-CTG TGG GCC AGG CCC GCC CCC GAA CTG CCT CCC TAC TGG-3'; M4 sense, 5'-CTA GGG CAG TCC GGG GGG CTT GCC GCA CAG A-3'; M4 antisense, 5'-CTG TGG GCC AGG CCC GCC CCC GAA CTG CCT CCC TAC TGG-3'). PCs were performed with the wild-type – 500/PAR1-Luc reporter vector as a template. The newly synthesized PCR products were digested with DpnI and used for transformation. The presence of mutations was verified by sequencing. Reporter vectors containing mutations were selected for large scale DNA preparations and used in transfection experiments.

**Transient Transfection and Luciferase Reporter Assay**—The cells were grown in 12-well plates to 80% confluence for at least 18 h and then transiently transfected using Lipofectin reagent (Invitrogen) with 1.5 μg of a firefly luciferase reporter gene and 50 ng of the Renilla luciferase reporter gene, driven by the β-actin promoter (Promega, Madison, WI). Cotransfection was performed by adding 1 μg of expression constructs to the DNA solutions. Luciferase activity was determined using the dual luciferase reporter assay system (Promega) in a microplate Luminoskan Ascent luminometer (Thermo Labsystems Inc., Franklin, MA) according to the manufacturer's instructions. Normalization of transfection efficiency was based on cotransfected β-actin Renilla luciferase activities.

### Results

**PAR-1 Expression in Human Melanoma Cell Lines Correlates with Metastatic Potential and Is Inversely Correlated with AP-2 Expression**—The cell lines in this investigation included primary and metastatic human melanoma cells with different tumorigenic and metastatic potentials in nude mice (34). Since the regulatory region of the PAR-1 gene contains several putative AP-2 binding sites, a panel of AP-2-negative and -positive melanoma cell lines was surveyed by RNase protection assay for differential expression of PAR-1. To further investigate the contribution of AP-2 in human melanoma, we reexpressed AP-2 in the human metastatic melanoma cell line WM266-4, which expressed negligible levels of endogenous AP-2. AP-2 expression, DNA binding activity, and promoter activation by AP-2 in the transfected cells were verified by reverse transcription-PCR, Western blot analysis, EMSA, and luciferase reporter assay (data not shown).

We observed a correlation between the level of PAR-1 expression and the metastatic potential of human melanoma cell lines. As shown in Fig. 1, high levels of PAR-1 mRNA were found in the metastatic melanoma cell lines A375SM and WM266-4 (lanes 1 and 2); on the other hand, low levels were found in the low and nonmetastatic melanoma MeWo and SB2 cell lines, respectively (lanes 3 and 4). The expression of PAR-1 was significantly lower in the WM266-4-AP-2-transfected cell lines C8 and C12A (lanes 5 and 6) than in the WM266-4 parental and nontransfected cell lines (lanes 2 and 3). Similar results were observed in the metastatic breast cancer cell line MDA-MB-231, which expressed high levels of PAR-1 (lane 7), whereas the nonmetastatic breast cancer cell line MCF-7 expressed undetectable levels of PAR-1 (lane 6). The PAR-1 mRNA amounts determined by densitometry, which was normalized to GAPDH expression, were ~4.5-fold higher in the metastatic melanoma cell lines (A375SM and WM266-4) than in the low and nonmetastatic melanoma cell lines (MeWo and SB2). In summary, PAR-1 was highly expressed in metastatic melanoma and breast cancer cell lines and expressed at significantly lower levels in nonmetastatic cell lines.

The RNase protection assay indicated that reexpression of AP-2 in WM266-4 cells resulted in decreased expression of PAR-1, which led us to further investigate the relationship

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This is a scientific excerpt from a research paper discussing the expression and regulation of PAR-1 in human melanoma cells. The text describes methods for quantifying PAR-1 and AP-2 expression using reverse transcription-PCR and luciferase reporter assays. The results indicate a correlation between PAR-1 expression and metastatic potential, with high PAR-1 levels found in metastatic cell lines and low levels in nonmetastatic cells. The paper also explores the role of AP-2 in regulating PAR-1 expression, showing that reexpression of AP-2 leads to decreased PAR-1 expression in metastatic cell lines. The findings suggest a potential therapeutic strategy for targeting PAR-1 in metastatic melanoma.
Metastatic melanoma cell lines WM266-4 and A377SM expressed high levels of PAR-1 transcripts, whereas low and nonmetastatic melanoma cell lines (MeWo and SB2) expressed low levels of PAR-1. Down-regulation of PAR-1 gene expression was observed in AP-2-transfected cells in comparison with the parental and neotransfected (Neo C5) cells. The metastatic breast cancer cell line MDA-MB-231 expressed high levels of PAR-1, whereas the nonmetastatic breast cancer cell line MCF7 did not express PAR-1. Metastatic potential in nude mice was as follows: H, high; L, low; N, nonmetastatic.

Similar results were obtained in breast carcinoma cell lines. The metastatic breast cancer cell line MDA-MB-231 expressed high levels of PAR-1 and undetectable levels of AP-2 (Fig. 2, A and B, lane 9), whereas the nonmetastatic breast cancer cell line MCF-7 expressed high levels of AP-2 and low levels of PAR-1. Reexpression of AP-2 in WM266-4 cells resulted in down-regulation of the PAR-1 mRNA and protein (Figs. 2, A and B, lanes 3 and 4).

In Vivo ChIP Analysis of the PAR-1 Promoter Demonstrates AP-2 and Sp1 Binding—The regulatory region of the PAR-1 gene has multiple putative AP-2- and Sp1-binding motifs, suggesting that these transcription factors probably mediate the promoter activity of PAR-1 (31, 32). To determine the roles of AP-2 and Sp1 in regulating the expression of PAR-1, we investigated whether these nuclear proteins were associated with the PAR-1 promoter in vivo, using the ChIP assay. Chromatin fragments from cultured cells were immunoprecipitated with an antibody to either AP-2 or Sp1, and DNA from the immunoprecipitates was isolated. From this DNA, a 276-bp fragment of the PAR-1 promoter region was amplified by PCR. This region of the promoter contained two AP-2-Sp1 complexes with multiple overlapping binding motifs for these transcription factors (Fig. 3A) (31). In cells that did not express AP-2 (WM266-4 and MDA-MB-231), Sp1 was predominantly bound to the PAR-1 promoter (Fig. 3B). In contrast, in cells that expressed AP-2 (WM266-4-AP-2-C8 and MCF-7), 9–10-fold more AP-2 was bound to the same region of the promoter (Fig. 3B). These data demonstrate that both AP-2 and Sp1 bind to the 3′ regulatory region of the PAR-1 promoter in a mutually exclusive manner.

AP-2 and Sp1 Bind to Complex 1 of the PAR-1 Promoter, Forming Distinct DNA-Protein Complexes—The in vivo analysis of the promoter by ChIP led us to further examine the two AP-2-Sp1 complexes within the PAR-1 promoter (Fig. 3A). To determine which nuclear proteins were bound to the regulatory region, we performed EMSA with oligonucleotide probes that corresponded to bp −365 to −329 (complex 1) and bp −206 to −180 (complex 2) of the 5′-flanking sequence of the PAR-1 gene (Table I) (31). To confirm the DNA binding activity of the nuclear proteins AP-2 and Sp1, nuclear extracts from WM266-4-AP-2-C8 cells were used in a gel shift assay that contained end-labeled double-stranded oligonucleotides of AP-2- and Sp1-binding motifs. Extracts from the WM266-4-AP-2-C8 cells revealed both AP-2 and Sp1 DNA binding to the AP-2 and Sp1 probes (data not shown). Incubation of the radiolabeled complex 1 (C1) probe with nuclear extracts from the WM266-4-AP-2-C8 cells resulted in the formation of four distinct shifted bands (labeled A, B, C, and D in Fig. 4A, lane 1). We also observed DNA-protein complexes migrating faster than these bands, as seen in Fig. 4A (lanes 5–7). The complexes appeared intermittently in our reactions but most often were present in the supershift analysis and, thus, might have represented complexes stabilized by the antibody or nonspecific interactions. The observed bands A, B, C, and D were identified in part by
competition with a 100-fold excess of unlabeled double-stranded oligonucleotides consisting of Sp1 and AP-2 consensus sequences. The binding of unlabeled Sp1 oligonucleotides inhibited the formation bands B, C, and D (Fig. 4, lane 3), whereas the binding of unlabeled AP-2 oligonucleotides inhibited the formation of band A (Fig. 4, lane 4).

To further define the nuclear proteins associated with complex 1, we performed supershift assays. When nuclear extracts were incubated with a specific anti-Sp1 antibody, bands B and C were diminished, and retarded (supershifted) complexes were formed, which indicates that these bands contained immunoreactive Sp1 protein (Fig. 4, lane 5). Incubation of the nuclear extracts with a specific anti-AP-2 antibody resulted in the loss of band A and the formation of a supershifted band (Fig. 4, lane 6).

To identify the nucleotide sequences required for AP-2 and Sp1 to bind to complex 1, we synthesized a series of mutated complex 1 oligonucleotides (Table I). The mutations were introduced to the overlapping AP-2 and Sp1 motifs and thus altered the nucleotides important for AP-2 and Sp1 to bind to the native sequence. We incubated radiolabeled mutant probes with nuclear extracts from WM266-4-AP-2-C8 cells. EMSA indicated that the mutations introduced in mutant probes eliminated band A and greatly affected the ability of AP-2 to bind to complex 1 (Fig. 4A, B, and C). The bindings of the nuclear factors Sp1 and Sp3 were also affected by mutations within the sequence of complex 1. The mutated probes M1 and M3 did not alter the formation of bands B, C, and D (Fig. 4A, B, and C), which coincided precisely with the DNA-protein complexes of complex 1 of the promoter (Fig. 4A). However, the mutant probes M2 and M4 completely abolished the formation of all DNA-protein complexes (data not shown), which suggests that AP-2, Sp1, and Sp3 bound to specific sequences within the PAR-1 promoter.

To further characterize the regulatory regions of the PAR-1 promoter, we performed EMSA using complex 2 (C2) as a probe and nuclear extracts from WM266-4-AP-2-C8 cells. Three major bands A, B, and C appeared (Fig. 4D). Competition and supershift assays confirmed the identity of distinct Sp1 and Sp3 DNA-protein complexes (Fig. 4D). In contrast, the formation of DNA-protein complexes with the sequence corresponding to complex 2 of the promoter did not involve AP-2 binding, which demonstrated binding specificity of AP-2 with complex 1 of the PAR-1 promoter. Taken together, the results presented in Fig. 4 demonstrate that AP-2, Sp1, and Sp3 bound to overlapping motifs in the complex 1 element of the human PAR-1 promoter in a mutually exclusive manner.

The 5’ GC Box (Complex 1) Is a Functional Element of the PAR-1 Promoter: Repression by AP-2 and Transactivation by Sp1—To define the functional elements responsible for PAR-1 gene regulation, we constructed a series of luciferase reporter plasmids containing serial 5’ deletions (Fig. 5A). These plasmid
constructs were transfected into a panel of melanoma cell lines that differentially express PAR-1. Using the β-actin-Renilla luciferase plasmid as a control, to normalize for transfection efficiency, we found that the promoter activity driven by the −1400/PAR-1-Luc construct was increased in A375SM and WM266-4 cell lines and decreased in WM266-4-AP-2-C8, SB2, and MeWo cell lines (Fig. 5B). Deletion of bp −1400 to −891 to create the −890/PAR1-Luc construct did not produce any marked differences in PAR-1 promoter activity (Fig. 5B), which implied that the elements in this region were not required for
**FIG. 5.** Luciferase reporter assay. A, schematic of the PAR-1 promoter luciferase constructs. Full-length PAR-1 promoter, bp −1400 to +1, and promoter deletions, bp −890 to +1 and bp −500 to +1, were cloned into the luciferase reporter construct pGL3. AP-2-like elements are depicted as small open ovals. Sp1 sites are shown as black circles. Complex 1 contains three AP-2 sites and five Sp1 sites and is depicted as a large gray oval. Complex 2 contains a single AP-2 site and two Sp1 sites and is shown as a striped oval. B, PAR-1 promoter luciferase activity in human melanoma cells. The human melanoma cell lines A375SM, WM266-4, WM266-4-AP-2-C8, SB2, and MeWo were transfected with full-length PAR-1 reporter constructs and deletion constructs. Luciferase activity was measured 36 h after transfection. Data shown are cumulative of three independent experiments performed in triplicate. C, WM266-4 and WM266-4-AP-2-C8 cells were co-transfected with PAR-1 deletion mutant reporter constructs and expression plasmids for AP-2, Sp1, or an empty vector (pcDNA3.1). Luciferase activity was measured 36 h after transfection. Data shown are cumulative of three independent experiments performed in triplicate. D, schematic representation of PAR-1 deletion mutant luciferase constructs. The base pair substitutions within the AP-2 and Sp1 binding sites of complex 1 are noted in boldface type and underlined. E, WM266-4 and WM266-4-AP-2-C8 cells were cotransfected with PAR-1 deletion mutant reporter constructs and expression plasmids for AP-2, Sp1, or an empty vector (pcDNA3.1). Luciferase activity was measured 36 h after transfection. Data shown are cumulative of three independent experiments performed in triplicate.
basal activity of the PAR-1 promoter. Further deletions of sequences between bp -890 to -501, which left the remaining 500-bp region of the promoter (−500/PAR1-Luc), increased PAR-1 promoter activity by 2-fold in A375SM and WM266-4 cell lines, whereas only a minimal effect was observed in WM266-4-AP-2-C8, SB2, and MeWo cell lines (Fig. 5B). This indicated that this 500-bp segment of the promoter is driven by a strong transcriptional activator. Furthermore, this 500-bp region of the promoter contains both complex 1 (bp −365 to −329) and complex 2 (bp −206 to −180), implicating these regulatory motifs in the regulation of this gene.

To further determine the effects of AP-2 and Sp1 on PAR-1 promoter activity, expression constructs containing cDNA encoding for AP-2 and Sp1 or an empty vector (pcDNA3.1-Neo) were transiently cotransfected with −500/PAR1-Luc construct in WM266-4 and WM266-4-AP-2-C8 cell lines (Fig. 5C). In comparison with −500/PAR1-Luc construct cotransfected with pcDNA3.1-Neo, transfection with −500/PAR1-Luc construct and pcDNA3.1-AP-2 resulted in a marked decrease in reporter gene activity in WM266-4 cells and a further decrease (1.5–2-fold) in luciferase activity in WM266-4-AP-2-C8 cells (Fig. 5C). In contrast, cotransfection of −500/PAR1-Luc construct with pcDNA3.1-Sp1 resulted in a marked increase in promoter activity by 2–2.5-fold. These results demonstrate that AP-2 functioned as a transcription repressor of PAR-1, whereas Sp1 was a strong transcriptional activator of PAR-1.

To assess the functional role of complex 1 (bp −365 to −329) in PAR-1 gene regulation, we performed site-directed mutagenesis within the Sp1 and AP-2 sites, similar to the mutations used in the gel shift analysis (Fig. 5D). Mutant luciferase reporter constructs were transiently transfected into the WM266-4 and WM266-4-AP-2-C8 melanoma cell lines, and their activity was compared with that of the −500/PAR1-Luc, which contained the native PAR-1 promoter sequences (Fig. 5E). Disruption of complex 1 with 2-bp mutations at the distal and middle regions (M1/PAR1-Luc) increased promoter activity in the WM266-4-AP-2-C8 cells by 1.4-fold, but no changes in luciferase activity were observed in WM266-4 cells compared with −500/PAR1-Luc activity. Cotransfection of M1/PAR1-Luc with AP-2 in WM266-4 and WM266-4-AP-2-C8 cell lines had no effect on promoter activity. Transfection with Sp1 resulted in a 2-fold increase in luciferase activity. Similarly, 2-bp mutations in the middle and proximal regions of complex 1 (M2/PAR1-Luc) decreased promoter activity by 0.5–2-fold in WM266-4 and WM266-4-AP-2-C8 cell lines. Introduction of AP-2 had no effect on promoter activity, whereas Sp1 had a minimal increase in the promoter activity of M2/PAR1-Luc. When 2-bp mutations were introduced in the distal and proximal region of complex 1 (M3/PAR1-Luc), we observed a 2-fold increase in promoter activity in WM266-4 and WM266-4-AP-2-C8 cell lines compared with −500/PAR1-Luc. Transfection with AP-2 had little effect on promoter activity, whereas Sp1 transactivated M3/PAR1-Luc. To our surprise, when 2-bp mutations were introduced simultaneously at the distal, middle, and proximal region of complex 1 (M4/PAR1-Luc), promoter activity was abolished in WM266-4 and WM266-4-AP-2-C8 cell lines, thus inhibiting the positive effect of Sp1 transactivation on complex 1 (bp −365 to −329).

The results of this targeted mutation analysis of the PAR-1 promoter, in conjunction with the deletion analysis and cotransfection with AP-2 and Sp1 expression plasmids, demonstrated that complex 1 was necessary for activation by Sp1 and repression by AP-2. Furthermore, when this region was significantly mutated, the promoter activity was lost, which suggests that complex 2 (bp −206 to −180) may have a minimal role in promoter activation.

The Ratio of AP-2 to Sp1 DNA Binding Activity and Expression Are Lower in Metastatic Melanoma Cell Lines—The presence of overlapping AP-2- and Sp1-binding motifs within the PAR-1 promoter suggests that AP-2 probably repressed expression of this gene by interfering with activated or basal transcription. Therefore, the levels of AP-2 and Sp1 DNA binding activity and expression in relation to the level of PAR-1 expression were examined by luciferase reporter assay and Western blot analysis. To determine the DNA binding activity of AP-2 and Sp1, we performed luciferase assays using either a reporter construct that contained three AP-2-binding sites (3×AP-2-Luc) or a vector that contained three Sp1-binding motifs (3×Sp1-Luc) in a panel of human melanoma cell lines (Fig. 6A). The data in Fig. 6B demonstrate that metastatic melanoma cell lines A375SM and WM266-4 had very low AP-2 binding activity in comparison with the low and nonmetastatic cell lines MeWo and SB2, which had significantly higher AP-2 activity. The AP-2 binding activity was 2.7-fold higher in WM266-4-AP-2-C8 cell line than in the parental WM266-4 cell line. Sp1 DNA binding activity was similar in the metastatic cell lines A375SM and WM266-4. The Sp1 activity was only slightly lower in SB2 cell line than in A375SM and WM266-4 cell lines. However, we observed a marked decrease in Sp1 DNA binding activity in the MeWo cell line. To calculate the ratio of AP-2 to Sp1 DNA binding activity, we used their relative luciferase activities. We determined that the metastatic cell lines A375SM and WM266-4 had a low AP-2/Sp1 ratio (−0.23), and the low and nonmetastatic MeWo and SB2 cell lines had significantly higher ratios of 3.1 and 1.4, respectively. The WM266-4-AP-2-C8 cells, in which AP-2 activity was restored, had a ratio equal to 0.68, which suggests that the balance of the transcriptional regulators determines the level of PAR-1 expression in melanoma cells. Similarly, the AP-2/Sp1 ratio was determined by Western blot and densitometry analyses (normalized to β-actin expression) (Fig. 6C). The metastatic melanoma cell lines A375SM and WM266-4 had an AP-2/Sp1 ratio of less than or approximately equal to 1 and expressed high levels of PAR-1 (Fig. 6C). In contrast, the low and nonmetastatic melanoma cell lines MeWo and SB2 had a ratio of AP-2/Sp1 greater than 1 and expressed minimal levels of PAR-1 (Fig. 6C). The WM266-4-AP-2-C8 cells displayed an increase in the ratio of AP-2/Sp1, which coincided with decreased PAR-1 expression. We conclude that the loss of AP-2 in melanoma cells resulted in a noticeable decrease in the ratio of AP-2 to Sp1 and correlated with high expression levels of PAR-1 and metastatic potential. Furthermore, reexpression of AP-2 restored the ratio of AP-2 expression to Sp1 expression in melanoma cells and resulted in down-regulation of PAR-1 (Fig. 7).

DISCUSSION

This investigation provides evidence that loss of AP-2 results in increased expression of PAR-1 in metastatic human melanoma cells, suggesting that PAR-1 overexpression contributes to the metastatic phenotype of melanoma. However, it should be noted that PAR-1 alone does not induce a metastatic phenotype; other malignant and invasive properties are also required. For example, Nierodzik et al. (28) reported that NIH/3T3 fibroblast cells, which expressed PAR-1 at low levels, and NIH/3T3 cells transfected to overexpress PAR-1 did not form pulmonary metastases after being intravenously injected into syngeneic mice. In a similar study conducted using B16F10 melanoma cells transfected with PAR-1, the metastatic potential increased 5–6-fold, demonstrating the need for already established metastatic properties of malignant cells (28). Even-Ram et al. (30) demonstrated that PAR-1 increased the invasive properties of human melanoma cells in vitro, primarily by...
promoting adhesion to extracellular matrix components. In their study, Even-Ram et al. (30) used the noninvasive human melanoma cell line SB2 transfected with PAR-1 cDNA and compared its properties to those of the highly invasive melanoma cell line A375SM. Indeed, SB2 clones expressing high levels of PAR-1 had an increased ability to invade, as was determined by Matrigel invasion assays. To that end, the invasiveness of the PAR-1-transfected SB2 cells was further enhanced when they were activated by either thrombin or the thrombin receptor-activating peptide that corresponds to the PAR-1 internal ligand SFFLRN. The invasive properties of the PAR-1-transfected SB2 cell line were explained, in part, by its preferential adhesion and specific recruitment of integrin α5β1 to focal contact sites accompanied by the cytoskeletal reorganization of F-actin toward migration-favoring morphology. Furthermore, PAR-1 probably contributed to metastasis by augmenting already established invasive properties of malignant cells. For example, the urokinase gene was induced by the activation of PAR-1 in human PC-3 prostate cancer cell line (41), resulting in the production of an enzyme required for tumor cell invasion (42, 43). These observations further implicated PAR-1 in the metastatic process of tumor progression and invasion.

The formation of new blood vessels is a critical determinant of tumor progression. Tumors are limited in size by their access to oxygen and nutrients from nearby blood vessels. Hence, solid tumors cannot exceed a diameter of 2 mm without the innermost cells undergoing necrosis (44). Thrombin is a potent promoter of angiogenesis via activation of PAR-1. Indeed, activation of PAR-1 in a variety of cell types can elicit a range of cellular responses and expression of thrombin-responsive genes. Many of the gene products are precisely those required for tumor angiogenesis and invasion, including interleukin-8 (45), vascular endothelial growth factor (46), basic fibroblast growth factor (47), platelet-derived growth factor (48), matrix metalloproteinase-2 (49), urokinase-type plasminogen activator (41), and αvβ3, αvβ5 integrins (30). This suggests that activation of PAR-1 may facilitate tumor invasion and metastasis by inducing the expression of cell adhesion molecules and matrix-degrading proteases and by stimulating the secretion of angiogenic factors. Taken together, these observations indicate a strong link between the cellular effects resulting from PAR-1 activation and the invasive properties of malignant melanoma.

Analysis of the PAR-1 promoter region revealed putative binding sites for many sequence-specific transcription factors, including Sp1, which is ubiquitously expressed, and AP-2, which is a cell type-specific transcriptional regulator. We noted with special interest the presence of multiple AP-2 consensus sequences in the PAR-1 promoter. AP-2 is a developmentally regulated and tissue-specific transcription factor expressed primarily in the neural crest (from which melanocytes are derived) and epidermal cell lineages. Although AP-2 was initially
described as a transcriptional activator, several recent studies have demonstrated its ability to behave as a repressor. In both the human acetylcholinesterase gene and the K3 keratin gene, there are overlapping Sp1 and AP-2 sites in the promoters, with Sp1 activating and AP-2 repressing gene transcription (52, 53). Indeed, our findings implicate Sp1 as a transcriptional activator and AP-2 as a transcriptional repressor of PAR-1 gene transcription. Although Sp1 is ubiquitously expressed, it is involved in the transcriptional regulation of a number of tissue-specific and differentiation-dependent genes, including genes that regulate neural, epithelial, and hematopoietic cell differentiation (52–54). A cooperation between ubiquitous and cell type-specific transcription factors is required to achieve precise differentiation (52–54). A cooperation between ubiquitous and cell type-specific and differentiation-dependent genes, including genes, all of which contribute to the malignant phenotype of human melanoma, are regulated by AP-2. Here we have identified PAR-1 as an additional AP-2 target gene that can play an active role in melanoma progression; overexpression and activation of PAR-1 in melanoma cells allows these cells to modulate the expression of genes involved in invasion and metastasis.

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Loss of Activator Protein-2α Results in Overexpression of Protease-activated Receptor-1 and Correlates with the Malignant Phenotype of Human Melanoma

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