Mechanism and biological significance of constitutive expression of MGSA/GRO chemokines in malignant melanoma tumor progression

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Abstract: By reverse transcriptase-polymerase chain reaction, enzyme-linked immunosorbent assay, and immunohistochemistry, MGSA-α, -β, -γ, and CXCR2 mRNA expression and proteins are detected in 7 out of 10 human melanoma lesions. The biological consequence of constitutive expression of the MGSA/GRO chemokine in immortalized melanocytes was tested in SCID and nude mouse models. Continuous expression of MGSA/GRO-α, -β, or -γ in immortalized melan-a mouse melanocytes results in nearly 100% tumor formation for each of the clones tested, whereas clones expressing only the neomycin resistance vector form tumors <10% of the time. Moreover, antibodies to the MGSA/GRO proteins slow or inhibit the formation of tumors in the SCID mouse model and block the angiogenic response to conditioned medium from the tumor-producing clones. Transcription of the MGSA/GRO chemokines is regulated by an enhancesome-like complex comprised of the nuclear factor-κB (NF-κB), HMG(I)Y, IUR, and Spl elements. In Hs294T melanoma cells the half life of the IκB protein is shortened in comparison to normal retinal epithelial cells, facilitating the endogenous nuclear localization of NF-κB. We propose that this endogenous nuclear NF-κB, working in concert with the 115-kDa IUR-binding factor, promotes constitutive expression of MGSA/GRO genes. *J. Leukoc. Biol.* 62: 588–597; 1997.

***Key Words:** CXC chemokines · MGSA/GRO proteins · angiogenesis · nuclear factor-κB · IκB*

INTRODUCTION

Melanoma growth stimulatory activity (MGSA) or growth-regulated proteins (GRO) along with interleukin-8, ENA-78, GCP-2, platelet factor-4, β-thromboglobulin, platelet basic protein, connective tissue activating factor, neutrophil activating peptide-2 (NAP-2), mig, interferon-γ-inducible protein-10 (IP-10), and stromal-derived factor-1 (SDF-1) comprise the CXC subfamily of chemokines [1–5]. There are three genes for MGSA/GRO that encode closely related mRNAs and proteins, MGSA-α, -β, and -γ [6–10]. Recently, we have identified a fourth human MGSA/GRO gene that is highly homologous but appears to encode a pseudogene [11]. The structure for the MGSA/GRO-α, -β, and -γ genes includes four exons, separated by three introns, which when spliced yield ~1.1 kb mRNAs (**Fig. 1**). The nucleotide sequences for these MGSA/GRO-β and -γ genes show 93 and 82% identity to MGSA/GRO-α, respectively. The amino acid sequences for these MGSA/GRO proteins are also highly conserved. Of the 73 amino acids of the mature peptide, the MGSA/GRO-β protein exhibits only seven amino acid differences for MGSA/GRO-α and the MGSA/GRO-γ protein exhibits only nine amino acid differences from MGSA/GRO-α. In the mouse, two homologs have been cloned, KC and macrophage inflammatory protein-2 [9, 12], and in rat the MGSA/GRO gene is named CINC [13]. Hamster, chicken, pig, sheep, and rabbit homologs have also been characterized [1, 10, 14–16].

EXPRESSION OF MGSA/GRO-α, -β, -γ IN MELANOMA TUMORS

To compare the expression of MGSA/GRO-α, -β, -γ genes during the clinical course of metastatic melanoma,

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Abbreviations: NF-κB, nuclear factor-κB; MGSA, melanoma growth stimulatory activity; GRO, growth-related proteins; NAP-2, neutrophil-activating protein-2; IP-10, interferon-γ-inducible protein-10; SDF-1, stromal cell-derived factor-1; RT-PCR, reverse transcriptase-polymerase chain reaction; IL-8, interleukin-8; RPE, retinal pigment epithelial cells; IRE, immediate upstream regulatory.

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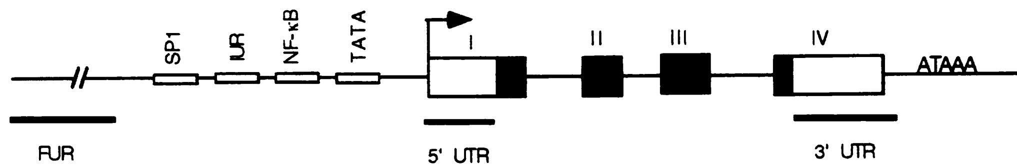

Fig. 1. Organization of the MGSA/GRO gene. The MGSA/GRO gene is organized into four exons (I through IV) interrupted by three introns. The 5′ regulatory region is characterized by four *cis*-acting elements that include binding sequences for the TATA binding factors, NF-κB, Sp1, and a novel element containing a TCGATC motif. The arrow indicates the site of transcription initiation. The region proximal to the transcription start site is highly conserved in α, β, and γ isoforms of MGSA/GRO. Significant differences exist in the far upstream (FUR) and the 3′ untranslated (3′ UTR) region of the three MGSA/GRO gene.

we examined the mRNA distribution of these three gene transcripts by reverse transcriptase-polymerase chain reaction (RT-PCR) in 10 human melanoma cases. The RT-PCR analysis detected all three MGSA/GRO mRNAs in 7 of 10 tumors, suggesting that all three MGSA/GRO genes may be transcribed endogenously in melanoma tumor cells (**Table 1**). By immunohistochemistry, we observed the expression of MGSA/GRO-α and CXCR2 protein in 7 of 11 melanoma cases studied (**Table 2** and **Fig. 2**) and in some of the tumors there was co-expression of CXCR2 (Table 2 and Fig. 2). By enzyme-linked immunosorbent assay the level of MGSA/GRO-α was considerably higher than MGSA/GRO-γ in most of the tumors studied (Table 1). The use of different portions of the tumor for ELISA, RT-PCR, and IHC studies produced some variability in the levels of MESA expression for these different assays.

In contrast to the expression of MGSA/GRO proteins, the angiogenesis inhibitory chemokine, IP10, was barely detectable in most of the melanoma lesions studied (Table 2 and Fig. 2) though it is abundantly expressed in normal skin. Thus a shift in the balance in expression of stimulatory and inhibitory CXC chemokines could play a part in the malignant progression of melanoma tumorigenesis, as has been suggested for non-small-cell lung cancer [17].

RECEPTORS FOR THE MGSA/GRO CHEMOKINE

Four receptors for MGSA/GRO, interleukin-8 (IL-8), and other ELR motif-containing CXC chemokines have been described: CXCR2, the *Herpesvirus samairi* receptor ECRF3, the Kaposi sarcoma human herpesvirus-8 G-protein-coupled receptor, and the Duffy antigen receptor for chemokines [ref. 4 and ref. 18 for review]. The receptor for the MGSA/GRO proteins is CXCR2, a seven-transmembrane G-protein-coupled receptor. CXCR2 binds not only MGSA/GRO, but also IL-8, NAP-2, GCP-2, and ENA-78 [10]. CXCR2 is present on neutrophils, basophils, lymphocytes, monocytes, keratinocytes, endothelial cells, melanocytes, and neurons [19–25]. Activation of this receptor affects cell migration as well as cell proliferation [10, 17, 24]. Receptor activation includes coupling of the trimeric G-protein complex that activates PLC-β, tyrosine kinases including

TABLE 1. Detection of MGSA/GRO and CXCR2 Expression by ELISA and RT-PCR

	ELISA		RT-PCR			
Case	MGSA-α (ng/mg protein)	MGSA-γ (ng/mg protein)	MGSA-α	MGSA-β	MGSA-γ	CXCR2
1	0.015	0.0	–	–	–	–
2	0.410	0.0	+	–	–	–
3	0.005	0.0	–	–	–	–
4	0.200	0.0	+	+	+	+
5	10.888	0.0	+	+	+	+
6	0.159	0.0	+	+	+	+
7	1.457	0.0	+	+	+	+
8	ND	ND	ND	ND	ND	ND
9	0.132	0.241	+	+	+	+
10	0.141	0.058	+	+	+	+
11	0.199	0.304	+	+	+	+

MGSA/GRO-α and MGSA/GRO-γ were measured by ELISA and reported as nanograms of MGSA per milligram of total protein from each human melanoma sample. Total RNA was extracted from melanoma tissues, reverse transcription was performed using an equal amount of RNA, then RT-PCR was performed using the same 5′ primer 5′-GCAGACCCTGCAGGGAATTC-3′, and using specific 3′ primer for MGSA/GRO-α 5′-GGCATGTTGCAGGCTCCTCA-3′, for MGSA/GRO-β 5′-ACCTCTCTGCTCTAACACAG-3′, and for MGSA/GRO-γ 5′-AGGTGGCTGACACATTATGG-3′.

ND, not determined; +, detectable; –, not detectable.

TABLE 2. Immunohistochemistry Detection of the Expression of MGSA/GRO-α, CXCR2, and IP-10 in Human Melanoma

Case	Antibody	Tumor cells	Endothelial cells	Macrophages	Other
1. MM	MGSA/GRO-α	some ++	−	−	−
Lymph node	CXCR2	−	−	−	−
	IP-10	−	−	−	CT±
2. MM	MGSA/GRO-α	−	−	−	−
Lymph node	CXCR2	++	−	−	F+
	IP-10	−	some +	−	−
3. MM/Liver	MGSA-α	−	−	−	−
	CXCR2	−	−	−	−
	IP-10	−	−	+	CT±
4. MM	MGSA/GRO-α	some ++	−	−	−
Lymph node	CXCR2	some ++	−	+	−
	IP-10	+	−	−	−
5. MM	MGSA/GRO-α	some +	some +	−	−
Lymph node	CXCR2	some ±	−	−	−
	IP-10	some ±	−	−	−
6. Secondary	MGSA/GRO-α	+++	−	++	F++
Cutaneous	CXCR2	some ±	some +	+	F+
Melanoma	IP-10	some ±	−	−	−
7. MM	MGSA/GRO-α	++	−	+	F++
Breast	CXCR2	−	−	−	−
	IP-10	−	−	−	−
8. Primary	MGSA/GRO-α	++	++	++	E++++
Melanoma	CXCR2	+++	+++	−	E++++
(Nevus)	IP-10	−	−	−	−
9. MM	MGSA/GRO-α	++++	++	++	L++
Lymph node	CXCR2	±	−	−	L+
	IP-10	+	±	−	CT++
10. MM	MGSA/GRO-α	−	+++	++	F+++
Leg	CXCR2	some ±	−	−	−
	IP-10	+	−	−	F++
11. MM	MGSA/GRO-α	−	+++	++	F+
Leg	CXCR2	±	−	−	SM+
	IP-10	±	−	+	CT±

Paraffin-embedded tissues were processed as in Figure 2 and immunostained with specific antibody at the dilution described in Figure 6.
±, slightly positive; +, positive; ++, moderately positive; +++, strongly positive; ++++, very strongly positive; CT, connective tissue; E, epidermis; F, fibroblasts; L, lymphocytes; MM, malignant melanoma; SM, smooth muscle.

p130CAS, and serine kinases [3, 22, 26, 27]. MAP-kinase is tyrosine phosphorylated as a result of this cascade [28]. Ligand binding to CXCR2 activates a serine kinase that phosphorylates the carboxy-terminal domain of the receptor itself, an event that regulates receptor desensitization and sequestration [27]. Recently, the small G-proteins rac and rho have been shown to be involved in CXC receptor cell migration [29] (**Fig. 3**). Our observation that melanoma tumor cells are expressing immunoreactive CXCR2 suggests that there is a potential for an autocrine loop such that the cells producing MGSA/GRO proteins can also respond to those proteins. We have previously reported that endothelial cells undergoing neovascularization in human burn wounds express immunoreactive CXCR2 [23]. However, others have had difficulty demonstrating the binding of IL-8 to human vascular endothelial cells [30]. We have observed that the endothelial cells of some of the microvessels in the melanoma tumors express immunoreactive CXCR2 (Tables 1 and 2, and Fig. 2). This opens the possibility that the MGSA/GRO expression can positively influence the growth of new blood vessels into the growing tumor and provide a continuous source of nutrients for the tumor.

MOUSE MODEL TO TEST THE ROLE OF MGSA/GRO PROTEINS IN MELANOCYTE TUMORIGENESIS AND ANGIOGENESIS ASSOCIATED WITH TUMOR GROWTH

To determine the biological significance of deregulation of MGSA/GRO expression in melanocytes expressing receptors for MGSA/GRO, we overexpressed MGSA/GRO-α, -β, or -γ in immortalized mouse melanocytes. Continuous expression of any one of these MGSA/GRO proteins enables these cells to form large colonies in soft agar and tumors in

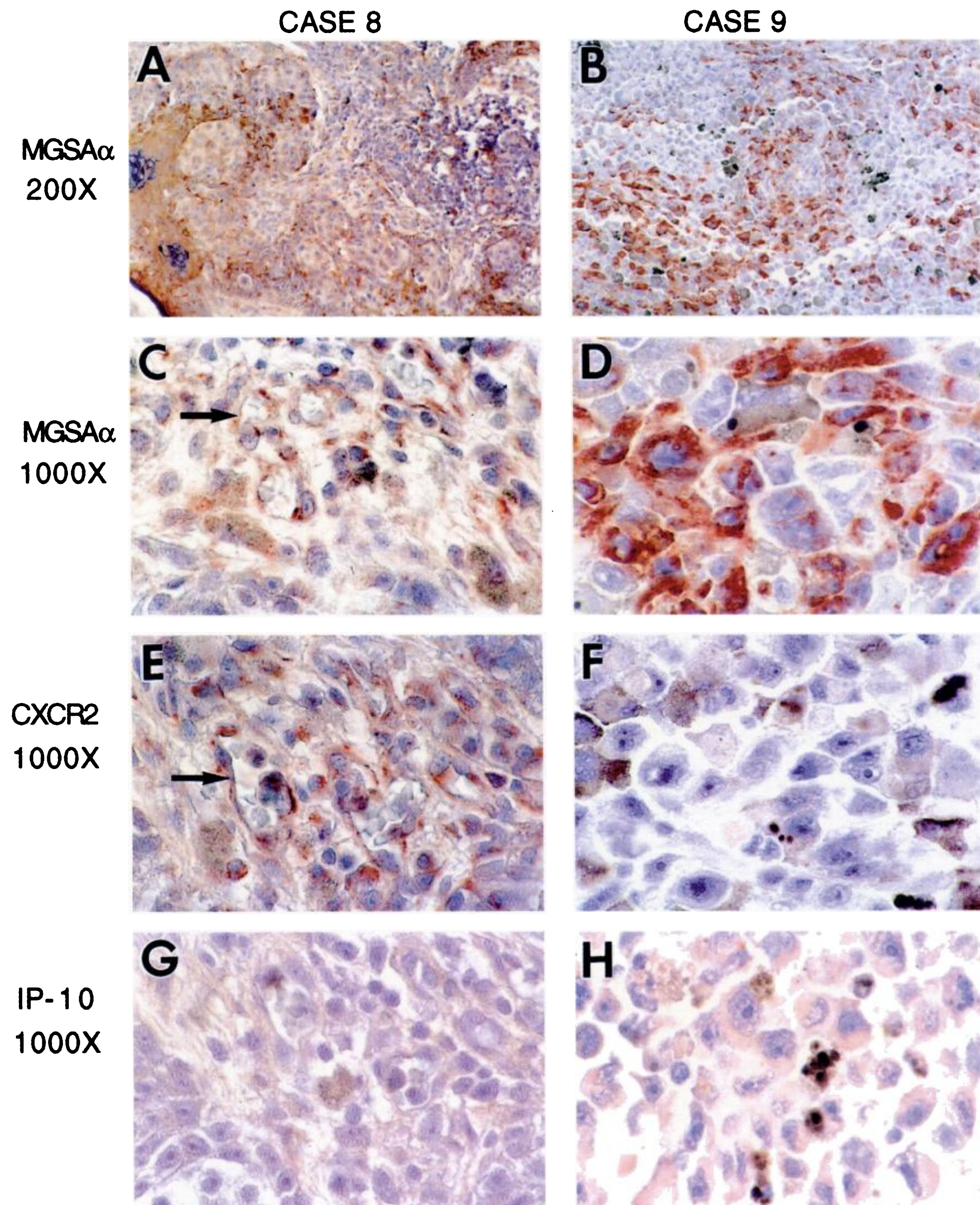

Fig. 2. Immunostaining of MGSA/GRO-α, CXCR2, and IP-10 in human melanoma. Human melanoma tumors were obtained at the time of surgery with informed consent according to NIH guidelines. Paraformaldeyde-fixed, paraffin-embedded tissue sections were examined for chemokines/chemokine receptor expression by indirect immunohistochemistry using VECTOR Laboratories ELITE ABC kit where immunodetection is visualized as a red or pink color. MGSA/GRO-α antibody [21] was used at a 1:100 dilution (panels A–D), CXCR2 antibody [21] 1:1000 dilution (panels E and F), and IP-10 antibody (a kind gift from R. Strieter) 1:500 dilution (panels G and H). In the nevus involved with primary melanoma shown in case 8 (panels A, C, E, G), MGSA/GRO-α and CXCR2 positive staining (red color) can be seen in tumor cells and microvessels (as indicated by the arrow), but IP-10 staining (pinkish red color) is barely detectable. In case 9 (panels B, D, F, H), tumor cells are stained strongly with MGSA/GRO-α antibody, lightly with IP-10 antibody, and barely detectable with CXCR2 antibody. In the absence of primary antibody no positive signal was observed (data not shown).

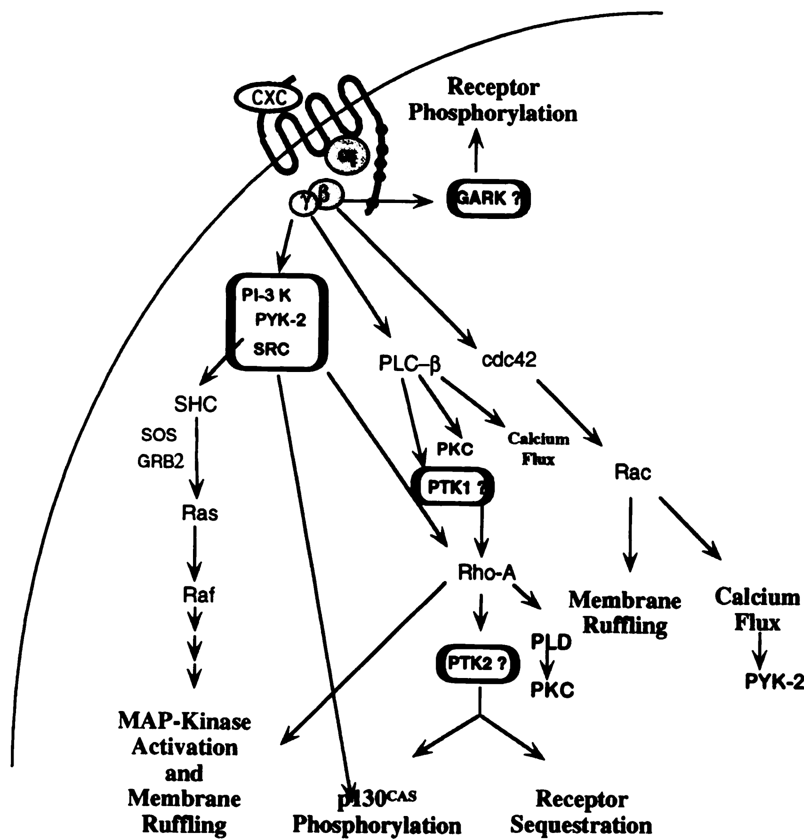

Fig. 3. Potential signal transduction pathways mediated by CXCR2.

nude mice, suggesting that these cells express a receptor for MGSA/GRO proteins [31, 32]. The resultant pigmented tumors have a histology compatible with that expected for melanoma based on S100 and HMB45 staining [31, 32]. MGSA/GRO proteins were produced by the melanoma cells in the tumor and the chemokine could be detected by immunohistochemistry. When cell cultures were established from the tumors and the neomycin-resistant colonies were again selected, these cells formed tumors a second time even more rapidly than with the initial injection. The tumors that formed were quite vascular, although not as much as those that form in the B16 melanoma controls. Conditioned medium from the MGSA/GRO-α, -β, and -γ expressing clones were examined for ability to promote angiogenesis with the use of the rat cornea micropocket assay [32]. Strong angiogenic responses were observed from conditioned medium from clones expressing each of the three MGSA/GRO proteins. Antibodies to the respective MGSA/GRO chemokines, but not normal rabbit serum, blocked that angiogenic response [32] (**Table 3**).

To test whether the tumor growth resulting in MGSA/GRO-expressing melanocytes could be inhibited with antibodies to the MGSA/GRO proteins, we injected SCID mice with melan-a clones overexpressing MGSA/GRO-α or MGSA/GRO-γ and injected antibodies to MGSA/GRO-α, antibodies to MGSA/GRO-γ, or normal rabbit serum into the initial cell inoculation site every other day over a period of two and one-half months. We observed that the antibody to MGSA/GRO-γ markedly suppressed tumor growth for the melan-a clones expressing MGSA/GRO-γ and only three of the six mice formed tiny tumor-like nodules. In contrast, the SCID mice carrying MGSA/GRO-γ-expressing proteins injected with normal rabbit serum rapidly formed large tumors. Antibody to MGSA/GRO-α only partially reduced tumor formation in the mice carrying MGSA/GRO-α expressing melan-a clones (**Table 4**). The number of viable endothelial cells in each tumor was determined by FACS analysis of Factor VIII positive cells. We are quantitating the number of endothelial cells in the tumors arising in SCID mice receiving MGSA/GRO expressing melanocytes and injections of antibody to MGSA/GRO as compared to the control group, which received injections of normal rabbit serum. These data demonstrate that the MGSA/GRO-α or MGSA/GRO-γ expression in immortalized melanocytes contributes to tumor growth by both autocrine and paracrine effects. The autocrine effects of MGSA/GRO promote the anchorage-independent growth of the cells and the paracrine effects promote the growth and migration of new blood vessels into the developing tumor. We predict that the receptor involved in these responses is CXCR2. Antibodies to CXCR2 have been shown to slow the growth of a variety of malignant melanoma cell lines in vitro [33]. Although the melan-a cells used in the assays described above express only low levels of MGSA/GRO receptor based on ligand binding assays, apparently these low levels of receptor are sufficient to transduce a

TABLE 3. Tumor Formation and Angiogenic Responses for MGSA/GRO-Expressing Melanocytes

MGSA-expressing cell line	Tumor formation	Nude mice Cornea angiogenesis responses: With conditioned medium	With NRS	With chemokine antibody
Mel-a-6	6/6	6/6	5/6	0/8
γ_3-14	7/9	6/7	5/6	1/5
γ_3-12	9/9	ND	ND	ND
γ_1-37	9/9	ND	ND	ND
β_2-19	9/9	4/6	5/6	1/6
β_2-5	9/9	3/6	ND	ND
β_2-13	9/9	ND	ND	ND
V-1	0/15	0/5	ND	ND
V-4	0/6	ND	ND	ND
V-6	2/6	2/6	ND	ND
Hs294T	5/5	ND	ND	ND

The MGSA/GRO-α-expressing clone (Mel-a-6), MGSA/GRO-β-expressing clones (β_2-5, β_2-13, β_2-19), MGSA/GRO-γ-expressing clones (γ_3-12, γ_3-14, γ_1-37), and vector control clones (V-1, V-4, V-6) were injected into nude mice. Tumor formation results are shown. Hydron pellets of serum-free culture medium concentrated from mel-a-6, β_2-19, γ_3-14, V-1, and V-4 were implanted into the rat cornea as previously described [17]. Angiogenic responses were shown by MGSA/GRO-expressing clones 6 days later and angiogenic responses were inhibited by antibodies to respective MGSA/GRO protein but not by normal rabbit serum (NRS).

biological response that results in tumor formation. However, we do not rule out the possibility that other proteins are also expressed in the MGSA/GRO overexpressing immortalized melanocytes, which may be induced secondarily and contribute to the transformation process. This hypothesis is currently under investigation. However, in the assays described above, three different melan-a clones expressing the neomycin resistance marker alone (vector control) were studied and of those controls only two tumors formed in 27 nude mice injected (Table 3); in the SCID model, no tumors were observed in the vector control group from the 12 mice injected (Table 4). Therefore, we propose that overexpression of MGSA/GRO proteins by immortalized melanocytes is a positive effector of melanocyte tumor progression.

TRANSCRIPTIONAL DEREGULATION OF MGSA/GRO GENE EXPRESSION

The transcriptional regulation of MGSA/GRO, IL-8, and CXCR2 gene expression requires multiple transcription factors. Sager's laboratory was the first to demonstrate that the nuclear factor-κB (NF-κB) element is an essential regulator of MGSA/GRO gene transcription [34]. Matsusaka et al. demonstrated that for IL-8, NF-κB works in concert with either C/EBP or AP-1 to modulate the cytokine-inducible transcription of IL-8 [35]. Sprenger et al. have shown that CXCR2 expression is induced by the cytokines, granulocyte-macrophage colony-stimulating factor and IL-2, and lipopolysaccharide can shorten the half life of the mRNA for CXCR2 [36]. We have concentrated our studies on elucidation of the mechanism for constitutive expression of chemokines in malignant melanoma tumor cells. The Hs294T human melanoma cell line expresses primarily MGSA/GRO-α mRNA endogenously, but MGSA/GRO-β and -γ are induced after lipopolysaccharide, IL-1, or tumor necrosis factor α treatment. The constitutive expression of the MGSA/GRO-α results in part from enhanced transcription in melanoma cells compared with normal retinal pigment epithelial cells (RPE) [37], and in part from enhanced stabilization of the mRNA for this gene [38]. We have shown that NF-κB is required for the cytokine-induced expression of MGSA/GRO genes as well as for the basal/constitutive expression of these genes. In addition to NF-κB, Sp1, HMG(I)Y, and the immediate upstream regulatory (IUR) elements/binding factors are required for the basal/constitutive expression of MGSA/GRO [39, 40]. Based on electromobility shift assay, there

TABLE 4. Inhibition of Melanoma Tumor Growth/Angiogenesis by Antibody to MGSA/GRO

MGSA-expressing cell line	Tumor formation: With NRS	Tumor formation: With antibody	SCID mice Mean tumor volume (cm^3): With NRS	SCID mice Mean tumor volume (cm^3): With antibody
Mel-a-6	6/6	5/6	1.1	0.7
γ_3-14	6/6	3/6	0.6	0.1
V-1	0/6	–	–	–
V-4	0/6	–	–	–

The MGSA/GRO-α-expressing clone (Mel-a-6), MGSA/GRO-γ-expression clone (γ_3-14), and vector control clones (V-1, V-4) were injected into SCID mice. Every other day these mice received an injection of 500 μL of antibodies to the respective MGSA/GRO protein at the site of melanocytes injection, and control groups received the same course of injection with 500 μL of normal rabbit serum (NRS). Tumor growth was followed by ruler measurement of palpatable tumors. At the time of death, total tumor volume was measured by water displacement and factor VIII positive cells infiltrating the tumor were quantitated by FACS analysis as previously described [17, 32]. The sample size was six for γ_3-14 with NRS, one for γ_3-14 with antibody treatment, five for mel-a-6 NRS and antibody treatment groups. The standard deviation for the γ_3-14 with NRS group was 14.6, for the mel-a-6 with NRS was 3.6, and for mel-a-6 with antibody was 12.1

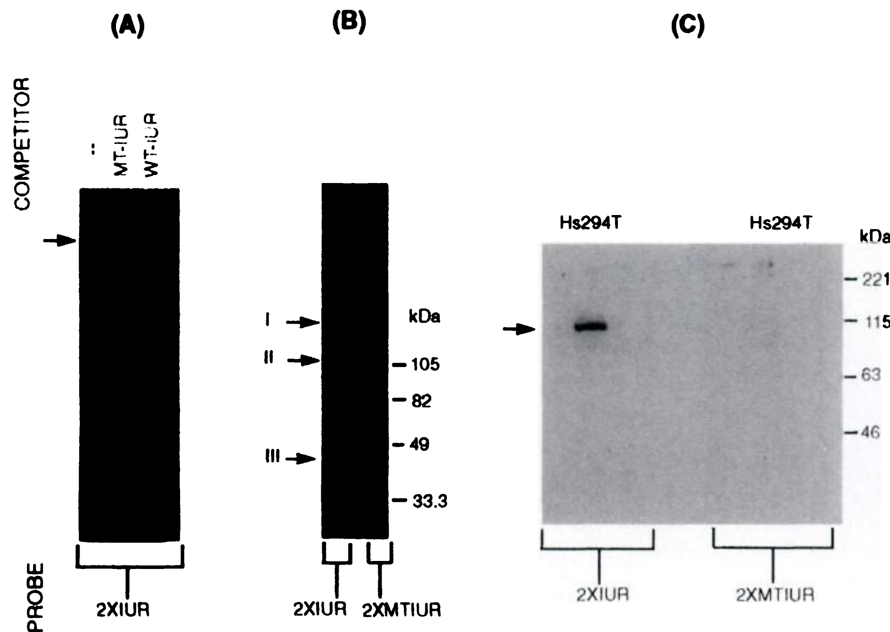

Fig. 4. Detection of IUR-F. Hs294T melanoma nuclear extracts were examined for sequence-specific binding to the IUR element. (A) Electrophoretic mobility shift assays (EMSA) with the IUR element show a single complex (arrow) that is competed by excess of wide type oligonucleotide (WT-IUR) but is unaffected by an excess of the mutant (MT-IUR) oligonucleotide. (B) UV-cross linking analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE): A 115-kDa protein (arrow) is observed as the major cross-linked product. Arrow II represents the 135-kDa, DNase I-undigested product. A third product (arrow III) at ~45 kDa can also be seen. (C) Southwestern blot analysis: Hs294T nuclear extracts were fractionated by SDS-PAGE, transblotted to membranes, and probed with either the IUR or the mutant IUR (MTIUR) elements. A 115-kDa protein binds to the IUR element but not the MTIUR element.

are no changes in the IUR binding factors, Spl, or HMG(I)Y in response to cytokine stimulation in RPE cells or Hs294T melanoma cells [39, 40].

RAPID TURNOVER OF IκB IN MELANOMA CELLS IN ASSOCIATION WITH NUCLEAR TRANSLOCATION OF NF-κB p65-p50 COMPLEX LEADS TO CONSTITUTIVE EXPRESSION OF MGSA/GRO

As we were searching to understand the mechanism by which melanoma cells constitutively express MGSA/GRO-α, we noted that in the absence of cytokine treatment there is pre-formed RelA p65 and p50 complex in the nucleus of Hs294T cells based on electrophoretic mobility shift assay (EMSA) [38]. By Western analysis, nuclei from the Hs294T cells exhibit significantly higher levels of RelA p65 than RPE cells. To discern the mechanism for this event, we examined the cytoplasmic levels of IκB in the two cell lines by Western blot. IκB protein levels were markedly lower in the cytoplasm of the Hs294T cells compared with RPE cells, which could in turn lead to enhanced transport of the active p65/p50 complex to the nucleus. However, the mRNA levels for IκB were not different in the two cell lines. This led us to examine the half life of IκB and through these experiments we learned that the IκB half life is significantly shorter in the melanoma cell line compared with the non-transformed RPE cells (**Table 5**). Thus the melanoma tumor cells have developed a mechanism for rapid turnover of IκB, which leads to an activated nuclear NF-κB complex, which can in turn induce the constitutive expression of chemokines and other factors that enable the cells to escape from apoptosis and become immortal.

TABLE 5. Stability of IκB Protein in Hs294T and ARPE Cells

CHX treatment (min)	IκB levels in Hs294T	IκB levels in ARPE (%)
0	100.0	100.0
15	51.6	86.6
30	56.6	91.6
45	31.6	106.6
60	26.6	80.0
90	18.3	63.3
120	13.3	46.6
150	23.3	43.3

Levels of IκB were measured by Western blot analysis of Hs294T and ARPE cytosolic extracts. The cells were treated for varying lengths of time with cycloheximide (CHX) so that stability of the protein in the two cell types could be studied. IκB degrades more rapidly in normal ARPE cells in contrast to Hs294T melanoma cells. Standard deviations of all values were in the range of ±10%.

CHARACTERIZATION OF THE IUR BINDING FACTOR

We have previously shown that an enhancer immediately upstream of the NF-κB element, the IUR, is also required for MGSA/GRO transcription [39]. We have shown that

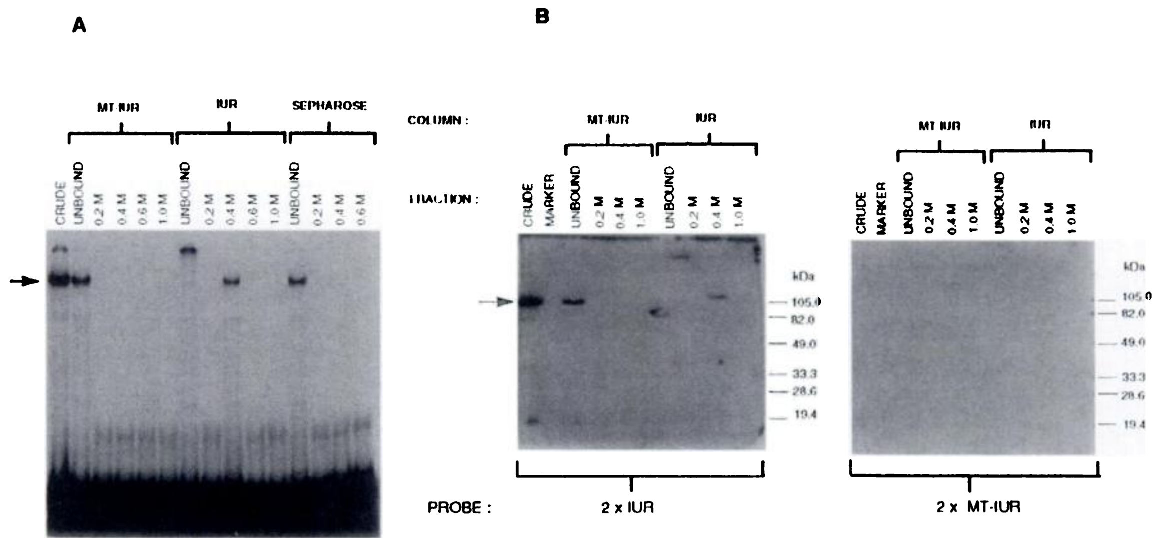

Fig. 5. (A) EMSA of DNA affinity chromatography fractions. Hs294T nuclear extracts were fractionated on Sepharose conjugated with mutant IUR (MTIUR) or wild-type IUR element with unconjugated Sepharose as control. Bound fractions were eluted with increasing concentrations of KCl. Flow through and eluates from all three columns were assayed for binding to the IUR element. IUR-F elutes in the 400 mM KCl fraction of the IUR but not the Sepharose alone or the MTIUR-Sepharose columns. (B) Southwestern blot analysis of affinity fractions. Bound and unbound fractions from the MT-IUR and IUR-Sepharose columns were fractionated by SDS-PAGE, transblotted to PVDF membrane, and probed with either the wild-type IUR or the mutant MTIUR elements. IUR-specific activity, migrating at a relative molecular size of 115 kDa, elutes in the 400 mM KCl fraction of the IUR-Sepharose column.

mutation of the IUR within the context of a 5′-350-bp MGSA/GRO-α/CAT reporter construct reduces the constitutive expression of the CAT reporter in both Hs294T and RPE cells and significantly reduces the IL-1 induction of CAT activity in the RPE cells [39]. Moreover, when a minimal 2× IUR reporter/CAT construct was transfected into Hs294T melanoma or RPE cells, it was capable of promoting the transcription of the CAT reporter [39]. The required nucleotide sequence for IUR binding was established through EMSA using a variety of mutants of the IUR as potential competitors of the labeled IUR probe [39]. With these experiments we were able to establish that the sequence TCGATC is essential for the EMSA by use of nuclear extracts from the RPE and Hs294T cells [39]. By ultraviolet-cross linking and Southwestern blot analysis we have demonstrated that the major protein binding to the IUR is ~115 kDa, with a minor band around 42 kDa. To further characterize the proteins binding to the IUR (IUR-F), we purified the IUR-F from Hs294T nuclear extracts by affinity chromatography using a multimerized IUR-oligonucleotide bound to Sepharose. The 115-kDa IUR-F eluted at 0.4 M NaCl from this affinity column and control columns prepared with mutant IUR oligonucleotide sequences did not bind the IUR-F (**Figs. 4 and 5**). We were able to demonstrate by both EMSA and Southwestern blot that the 115-kDa protein elutes from the column at this position. Con-

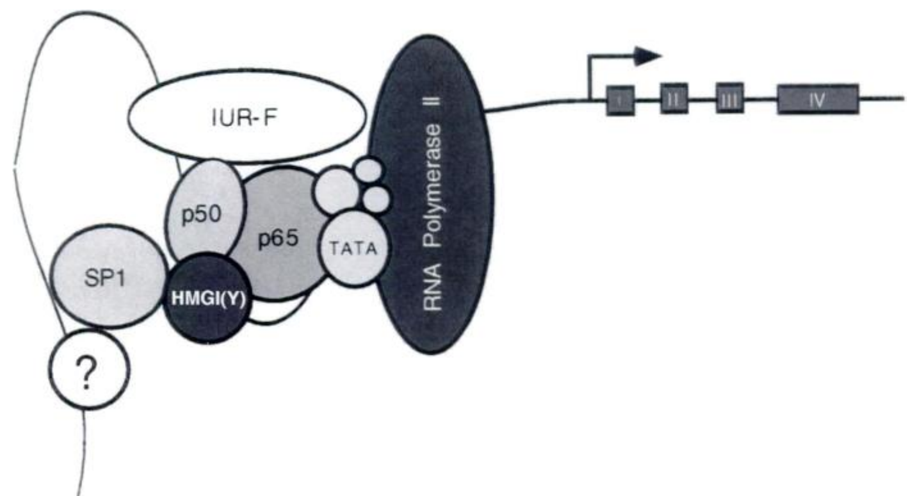

Fig. 6. Transcriptional regulation of the MGSA/GRO gene, Putative Enhanceosome. Five *cis*-acting elements have been identified in the MGSA/GRO 5′ regulatory region. A model for the transcriptional regulation of the gene includes interaction of RelA (p65) and p50 subunits of NF-κB with other components of an enhanceosome involving the TATA box, HMG(I)Y element, an SP1 element, and a novel element, IUR, which contains a TCGATC motif. Additional factor(s) binding further upstream in the 5′ regulatory region may also play a role in the regulation of MGSA/GRO gene expression.

trols using mutant IUR in both Southwestern blot and EMSA demonstrate that the binding of the 115-kDa protein is specific for the TCGATC sequence.

Initial sequence attempts on preparations of IUR-F transblotted to PVDF membranes suggested that this protein is blocked at the amino terminus. Therefore we turned to an alternate approach of the one hybrid screen using the IUR as bait with prey cloned from a cDNA expression library where the expressed proteins were fused to the Gal-4 activation domain. With this system we identified several IUR-F candidate clones. The IUR element is found not only in the MGSA/GRO gene promoter, but also in the CXCR2 promoter, and in the promoters of several cytokines and thus is likely to be a strategic regulator of transcription.

CONCLUDING REMARKS

In this review, we have detailed experiments that revealed the relative frequency of the expression of the MGSA/GRO proteins and the CXCR2 receptor for these chemokines in human melanoma tumors, the biological significance of continuous expression of MGSA/GRO, and the mechanism for endogenous expression of MGSA/GRO in melanoma cells. Thus, conditions leading to continuous expression of ELR containing CXC chemokines can potentiate tumor growth. This potentiation appears to involve both autocrine effects on the melanocytes and paracrine effects on the endothelial cells. The constitutive expression of MGSA/GRO genes is regulated by at least four transactivating factors: NF-κB, HMG(I)Y, Sp1, and the element immediately upstream of the NF-κB element, the IUR (**Fig. 6**). Hs294T malignant melanoma cells exhibit a rapid turnover of the inhibitor of nuclear NF-κB, known as I-κBα. We postulate that factors such as ultraviolet irradiation, chronic inflammation, and chemical carcinogens can lead to the endogenous activation of kinases responsible for the phosphorylation of I-κB. As a result of this phosphorylation, I-κB is degraded, NF-κB is translocated to the nucleus, and the resultant activated NF-κB works in concert with the IUR, Sp1, and HMG(I)Y elements/binding factors to produce the constitutive expression of the MGSA/GRO genes. This constitutive expression of chemokine in cells that also express receptor for MGSA/GRO is associated with tumorigenesis.

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