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Mechanisms Regulating c-met Overexpression in Liver-Metastatic B16-LS9 Melanoma Cells

Giuliano Elia,[1] Yuan Ren,[1] Patrizia Lorenzoni,[1] Reza Zarnegar,[2] Max M. Burger,[1] and Dario Rusciano[1]*

[1]Friedrich Miescher Institute, Novartis Research Foundation, Maulbeerstrasse 66, CH-4058 Basel, Switzerland
[2]Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261

Abstract Liver selected B16-LS9 melanoma cells show a dramatic overexpression of the proto-oncogene c-met, the cellular receptor for hepatocyte growth factor/scatter factor. As a consequence, c-met becomes constitutively active, and the cells become more responsive to hepatocyte growth factor stimulation. We have investigated the molecular mechanisms regulating c-met expression in both the parental line B16-F1, which has low expression levels, and the liver-specific B16-LS9, overexpressing c-met. Overexpression is observed at the protein and mRNA levels, however without further evidence of gene amplification or rearrangement. c-met promoter activity was higher in B16-LS9 than B16-F1 cells, and also a nuclear run-off showed higher transcription levels in B16-LS9 cells. Moreover, we found that c-met mRNA had a longer half-life in B16-LS9 cells, thus indicating also the involvement of post-transcriptional regulation mechanisms. Finally, we found evidence that autonomous activation of the melanocortin receptor-1 (MCR-1) is at least partially responsible for c-met upregulation in B16-LS9 cells, since treatment of the cells with a potent MSH antagonist (the agouti peptide) has strong down-regulatory effects. J. Cell. Biochem. 81:477–487, 2001.

Key words: B16 melanoma; c-met; HGF/SF; MSH; agouti

Cancer metastasis represents the major cause of morbidity and death for cancer patients. Indeed, it is the peculiar ability of malignant tumor cells to escape from the primary tumor and settle at distant organ sites, coupled with their genetic instability, which makes tumors such a difficult subject for any kind of direct therapy. The metastatic phenotype results from multiple and complex changes within the tumor cell, which cannot be attributed to just one single gene [Rusciano and Burger, 1993]. However, some of the properties that are required by cancer cells to become metastatic can be conferred by the activation of the proto-oncogene c-met, the cellular receptor for hepatocyte growth factor/scatter factor (HGF/SF) [Bottaro et al., 1991]. In fact, upon activation, c-met starts a cascade of events that results in pleiotropic effects on cell behavior, including regulation of growth, motility and invasion [Rusciano et al., 1998a; Weidner et al., 1993], all of which are required for the successful production of the metastatic phenotype [Bardelli et al., 1999; Jeffers et al., 1996; Rong et al., 1994].

Overexpression of the proto-oncogene c-met is a fairly common event in many human cancers [To and Tsao, 1998], where it has been correlated with the malignant properties of tumor cells [Vande Woude et al., 1997]. For instance, in human melanomas tumor progression from the radial to the vertical, invasive growth phase tightly correlates with overexpression of c-met [Natali et al., 1993]. Similarly, c-met too has a prominent role in the malignant behavior of many murine experimental tumors [Jeffers et al., 1996; Meiners et al., 1998; Rong et al., 1994]. In transgenic mice, expression of a mutationally activated form of c-met resulted in the induction of metastatic mammary adenocarcinomas [Jeffers et al., 1998], whereas ectopic expression of HGF/SF induced the spontaneous formation of a number of epithelial and mesenchymal tumors, among which predominated cutaneous melanomas [Otsuka et al.,

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*Correspondence to: Dario Rusciano. Research Center, Sifi SpA, Via Ercole Patti 36, 95020 Lavinaio, Catania, Italy. E-mail: dario_rusciano@sifi.it

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1998]. Interestingly enough, some of these melanomas overexpressed a normal form of c-met, and were metastatic to the liver. This is in keeping with our previous findings with the B16 mouse melanoma model system, in which we described a tight correlation between c-met overexpression and constitutive activation, and growth as liver metastasis [Lin et al., 1998; Rusciano et al., 1995, 1996]. Clearly, c-met overexpression in liver-selected B16-LS9 cells resulted in a higher response of these cells to HGF/SF in terms of growth, motility and invasion [Rusciano et al., 1998a]. Moreover, we have recently described the involvement of c-met and HGF/SF in the differentiation process of B16 cells. We found that melanocyte-stimulating hormone (MSH), the physiological inducer of differentiation for melanocytes, was also a potent inducer of c-met expression in B16 cells [Rusciano et al., 1999]. On the other hand, we showed that HGF/SF treatment of B16 cells led to a dramatic decrease of tyrosinase expression, which in turn translated into a diminished synthesis of melanin [Rusciano et al., 1998b].

The question that we address in this work concerns the mechanisms underlying the overexpression of c-met in liver-selected B16-LS9 melanoma cells. Results reported here indicate that both transcriptional and post-transcriptional events regulate the amount of specific messenger found in the cells. We also show that induction of differentiation by MSH treatment enhances c-met promoter activity, and present evidence that an increased activity of the MCR-1 strongly contributes to c-met overexpression in B16-LS9 cells.

MATERIALS AND METHODS

Cell Culture

B16 melanoma cell lines were routinely cultured in DMEM with 10% FCS and antibiotics (penicillin and streptomycin) in a humidified incubator at 37°C and 10% CO_2. Subculturing was done two to three times per week, detaching the cells by a short (5 min) incubation at 37°C in Ca^{2+}- and Mg^{2+}-free PBS (CMF-PBS) containing 0.02% EDTA. Cell cultures were free of mycoplasma contamination, as detected by Hoechst staining. MSH (Sigma) and agouti (a kind gift of Dr. DH Willard) were added to tissue culture medium at the indicated concentrations to analyze their effect on c-met expression.

Cell Extracts and Western Blotting

B16 cells grown as monolayers were extracted by 20-min incubation with cold CHAPS buffer ($MgCl_2$ 5 mM; EGTA 1 mM; NaCl 100 mM; glycerol 10%; CHAPS 1% w/v; HEPES 25 mM, pH 7.4) containing orthovanadate 1 mM and protease inhibitors (25 μg/ml of both aprotinin and leupeptin, Sigma; 1 mM AEBSF, Calbiochem). Extracts were clarified by centrifugation, diluted with reducing Laemmli's sample buffer and boiled. Proteins were determined in separate aliquots by the DC-Lowry assay (Bio Rad). Similar amounts of proteins were separated by SDS-PAGE and immunoblotted onto PVDF membranes (Immobilon, Millipore) with specific antibodies (anti-phosphotyrosine monoclonal antibody 4G10 was a kind gift of Kurt Ballmer, FMI; rabbit polyclonal antibody against c-met was obtained from Santa Cruz Biotechnology). Peroxidase-labeled secondary antibodies (DAKO) were detected by the luminol reaction (Amersham) on Hyperfilm (Amersham).

Northern and Southern Blotting

Poly A + RNA was prepared from confluent dishes by using the Micro mRNA purification kit (Pharmacia) according to the manufacturer's instructions. Two micrograms of RNA was run on a 0.8% formaldehyde/agarose gel, and blotted to a nylon membrane (Boehringer Mannheim). Methylene blue staining of the membrane was used to check loading of the lanes. High molecular weight genomic DNA was prepared from confluent monolayers of B16 cells by the procedure of Blin and Stafford [Blin and Stafford, 1976] as modified by Maniatis [Sambrook et al., 1989]. After digestion with Hind III and Bam HI, 20 μg of DNA was separated on agarose gel and blotted to the nylon membrane following the procedure described by Maniatis [Sambrook et al., 1989]. The murine-specific probe for c-met (2.2 kB) was kindly provided by Guido Hartmann (MDC, Berlin) and radioactively labeled with α^{32}P-dCTP (Amersham) by the Random Primed DNA Labeling Kit (Boehringer Mannheim).

Promoter Activity

Three different lengths (0.3, 0.5 and 1.6 kb) of the cloned promoter region of c-met [Seol and Zarnegar, 1998] were inserted into the pCAT basic reporter vector (Promega). Empty pCAT

basic and pCAT-3 containing the SV40 promoter were used, respectively, as negative and as positive control, and the latter also as reference to check the transfection efficiency. Thereafter, 2 μg of pure plasmid DNA was transfected into sub-confluent cells using the lipofectamine-based method as described by the manufacturer (Gibco-Life Technologies). Forty-eight hours after transfection cell lysates were prepared following the indications of the pCAT-ELISA reagent kit (Roche Biochemicals), and quantitation of pCAT expression carried out according to manufacturer's instructions. To detect the effects of MSH or agouti on c-met promoter activity, cells were treated with the respective peptide immediately after the transfection procedure (which lasted 6 h), and until the end of the experiment at 48 h.

Transcriptional Run-On Assay

In vitro run-on transcription assay was performed as follows. Intact nuclei were isolated from 5×10^7 B16-F1 and B16-LS9 mouse melanoma cells grown under identical conditions. Cells were scraped from the monolayer with a rubber policeman, washed twice with ice-cold PBS, resuspended in 4 ml lysis buffer (10 mM Tris–HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP_{40}) and kept on ice for 5 min. The pellets were resuspended in 100 μl of nuclei storage buffer (50 mM Tris–HCl pH 8.0, 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA), snap-frozen in liquid nitrogen and stored at −80°C. For the transcription reaction, nuclei were thawed by addition of the same volume (100 μl) of a twofold concentrated reaction mixture containing 10 mM Tris–HCl pH 8.0, 5 mM $MgCl_2$, 0.3 M KCl, 1 mM DTT, 0.2 mM EDTA, 4 mM each of ATP, GTP, CTP and 100 μCi of [^{32}P]UTP (Amersham, 3000 Ci/mM). Samples were incubated at 37°C for 30 min. Reactions were stopped by addition of 10 μl of RNAse-free DNAse I (Amersham, 10,000 units/ml) for 30 min at room temperature. Samples were then solubilized by the addition of 600 μl of a buffer containing 10 mM Tris–HCl pH 8.0, 2% SDS, 7 M urea, 0.35 M LiCl, 1 mM EDTA, 100 μg carrier tRNA, and incubated for 2 h at 45°C in the presence of proteinase K (Sigma, final concentration 1 mg/ml). Nucleic acids were precipitated by adding ice-cold TCA to 10% final concentration, incubating on ice for 20 min, and spinning for 20 min at 4°C in an Eppendorf refrigerated centrifuge at the maximum speed. Pellets were washed twice in cold ethanol to remove any trace of TCA and resuspended in 100 μl TES (10 mM Tris–HCl pH 8.0, 1 mM EDTA, 0.5% SDS). 10^6 d.p.m. of each radiolabeled, in vitro transcribed RNA sample was then hybridized to nylon-immobilized (Hybond-N+, Amersham) plasmids containing a 2.2-kb fragment of c-met, a full length c-met, the glyceraldehyde phosphate dehydrogenase gene (GAPDH) or the empty vector (BSSK-), for 72 h at 55°C in a hybridization buffer (50% formamide, 6 × SSC, 10 × Denhardt's, 0.2% SDS). After stringency washings, strips were exposed to X-ray films and the bound radioactivity was quantitated by molecular dynamics phosphor imager analysis.

mRNA Stability Assay

Replicas of semi-confluent cultures of B16-F1 or B16-LS9 mouse melanoma cells were treated with actinomycin D (Sigma, final concentration 5 μg/ml). At different times after actinomycin D addition (0, 2, 4, 6 and 8 h), dishes were washed twice with ice-cold PBS, and cells collected by scraping with a rubber policeman. Cells were then centrifuged and poly-A^+ mRNA was purified by the QuickPrep Micro mRNA purification kit (Pharmacia), following the protocol suggested by the manufacturer. Poly-A^+ mRNA molecules were then separated on 0.8% agarose-formaldehyde gels and blotted to Nylon membranes (Hybond N+, Amersham) as above. A digoxygenin-labeled (DIG) c-met specific RNA probe was transcribed by a DIG-RNA labeling kit (Boehringer) and using a c-met 2.2-kb fragment-containing, linearized plasmid as a template. After a 2-h pre-hybridization at 68°C in 50% formamide, 5 × SSC, 10 × Denhardt's, 0.1% Na lauryl sarcosinate, 0.02% SDS, mRNA was hybridized overnight at 68°C in the same buffer containing 50 ml of the DIG-labeled RNA probe. Detection of DIG-RNA was performed by use of an anti-DIG antibody conjugated to alkaline phosphatase (Boehringer) and Lumigen PPD chemiluminescence detection kit (Boehringer), following the manufacturer's instruction. For normalization of the results, filter was then stripped and re-hybridized to a β-actin DIG-RNA probe, prepared as above.

Statistical Analysis

The *t*-test for independent samples has been applied to results reported in Figure 3. All the other experiments illustrated in the other fig-

ures have been performed at least three times, and results from an experiment most indicative of the observed trend are reported in the figure.

RESULTS

We have previously shown that liver-selected B16-LS9 cells express abnormally high amounts of the product of the c-met proto-oncogene, the cellular receptor for HGF/SF, which then becomes constitutively active in consequence of its elevated density on the plasma membrane [Rusciano et al., 1995, 1996]. Figure 1 illustrates a comparison between the parental, unselected B16-F1 cell line, and B16-LS9 cells. From the Western immunoblot analysis (Fig. 1A) it is evident that c-met protein is dramatically overexpressed in B16-LS9 cells (lower panel), and that it is heavily phosphorylated on tyrosine (upper panel), which is indicative of constitutive activation [Bardelli et al., 1992; Ferracini et al., 1991]. Protein expression clearly correlates with the amount of mRNA expressed in the two cell lines (Fig. 1B). However, the pattern observed after Southern blotting (Fig. 1C) is identical for B16-F1 and LS9 cells: two main bands of similar size and intensity are visible in both cell lines, indicating no further gene amplification or rearrangements that could explain the increased amount of specific mRNA detected in B16-LS9 cells.

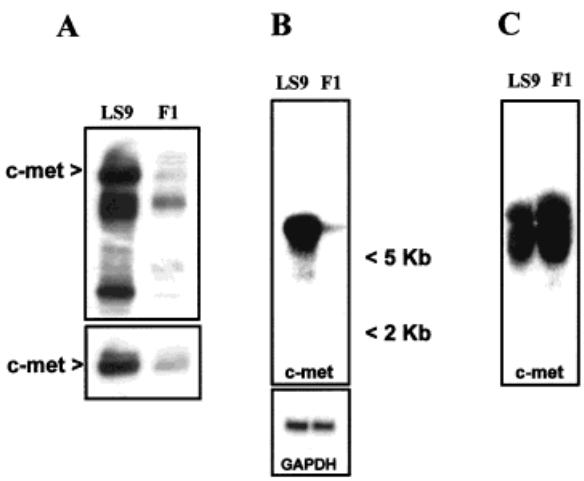

Fig. 1. C-met analysis in B16-LS9 and B16-F1 cells. **A:** Western blot analysis: 30 μg of total cell extracts was separated on a 6% SDS-PAGE under reducing conditions, blotted to PVDF and immunostained with anti-phosphotyrosine monoclonal antibody (upper panel), or with anti-met polyclonal antibody (lower panel). The identity of the phosphorylated band at 145 kDa as the β-chain of c-met was demonstrated previously (Rusciano et al., 1995). **B:** Northern blot analysis of 2 μg of poly-A^+ mRNA. The lower panel shows GAPDH bands as control for loading. **C:** Southern blot analysis of 20 μg of genomic DNA after digestion with Hind-III and Bam-HI.

Transcriptional Regulation

In order to investigate whether the higher level of c-met mRNA present in B16-LS9 cells was due to increased transcription of the gene, we performed a nuclear run-off experiment, comparing the amount of specific transcript in B16-F1 and B16-LS9 cells. Results reported in Figure 2 clearly indicate that c-met transcription was much more active in liver-selected B16-LS9 cells than in the parental B16-F1 cell line, as well as when the values were corrected for the different intensity of GAPDH, used as internal standard (see bottom panel). Similar results, however more quantitative, were provided by an experiment in which mouse c-met promoter activity was measured by the CAT reporter gene assay. Three different lengths of the mouse c-met promoter [Seol and Zarnegar, 1998] were subcloned into the pCAT basic vector and transfected into B16 cell lines. Empty vectors, or pCAT3 in which CAT transcription is led by the SV40 promoter, were used as controls. Results reported in Figure 3 clearly show that the intermediate promoter length has the highest activity in both cell lines, indicating the presence of repressor elements within the deleted upstream 1.1 kb sequence, and that the 0.3 kb promoter has the least activity, which appears to be similar in both B16-F1 and B16-LS9 cells. This indicates that the relevant regulatory elements are present within the distal 1.3 kb segment. Similar results had already been reported for the mouse promoter with C33-A epithelial cells [Seol and Zarnegar, 1998], and for the human c-met promoter [Gambarotta et al., 1994]. In B16 cell lines the two longer elements (1.6 and 0.5 kb) were definitely, even though not dramatically, more active in B16-LS9 than in B16-F1 cells, thus confirming that an enhancement of transcription contributes toward the increased amount of c-met mRNA observed in the liver-specific B16 cell line.

Post-Transcriptional Regulation

The amount of any specific mRNA found anytime in a cell is the combined result of the transcriptional activity of the relative gene and the stability of the message once exported in the cytoplasm. Therefore, we set out to compare c-met mRNA stability in B16-F1 and B16-LS9

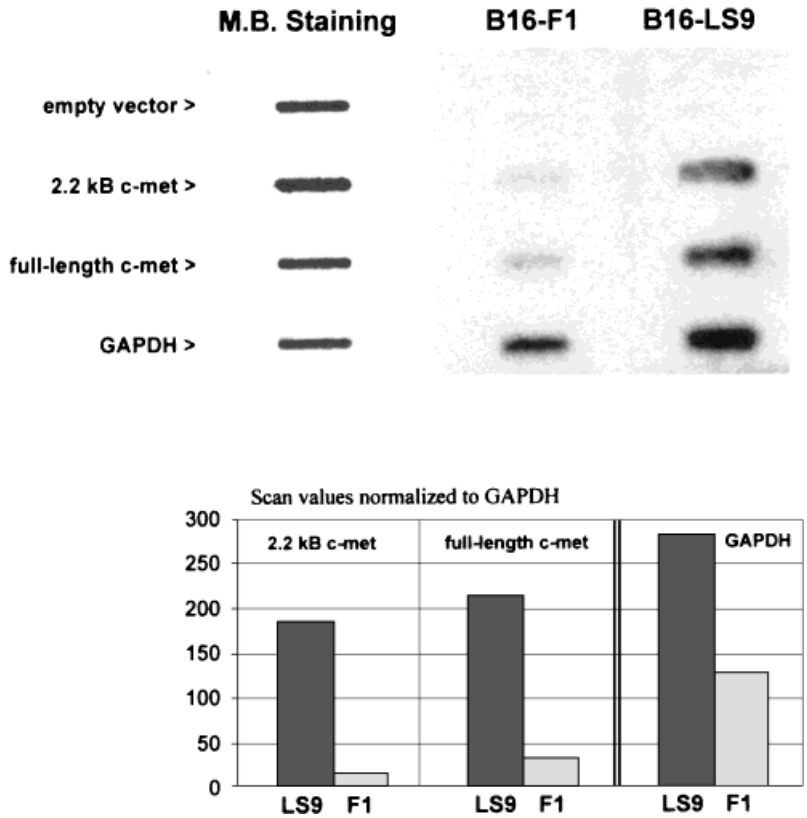

Fig. 2. Nuclear run-on assay. The left lane reports the methylene blue staining of the immobilized DNA fragments, to show the relative loading. Two different lengths of c-met cDNA have been used. The bottom panel shows a densitometric analysis of the results, with the respective values for B16-F1 and B16-LS9 normalized to GAPDH for direct comparison.

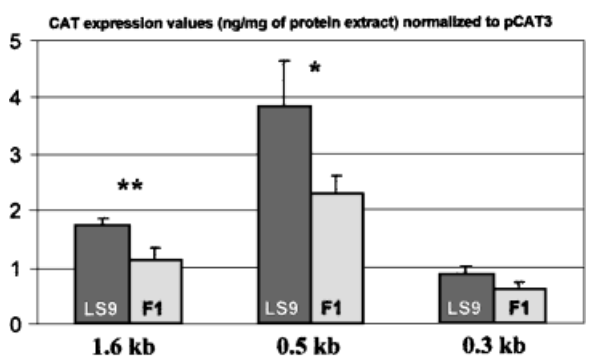

Fig. 3. Quantitation of c-met promoter activity in B16 cell lines. Three different lengths of the mouse promoter for c-met (1.6, 0.5 and 0.3 kb) in the pCAT reporter plasmid have been transiently transfected by the lipofectamine method. Empty pCAT vector and pCAT3 (in which pCAT expression is led by the SV40 promoter) served as controls. CAT expression values were measured with an ELISA method, and normalized to the values obtained in either cell line with pCAT3 (empty pCAT vector gave negligible optical readings) for direct comparison. Results from three different representative experiments have been averaged, and standard deviation is reported. Asterisks on top of the bars indicate the significance of the data, as evaluated by the *t*-test: * $P \leq 0.05$; ** $P \leq 0.01$.

cells. Actinomycin-D treatment to prevent new mRNA synthesis affected only marginally the stability of the β-actin mRNA message, known to be among the most stable in many cells (Fig. 4B). C-met message appeared to be similarly stable in both B16 cell lines until 4 h of treatment, beyond which time there was a sudden drop in B16-F1, while the message remained fairly stable in B16-LS9, thus indicating also a contribution of mRNA stability to c-met overexpression in B16-LS9 cells.

Mechanisms Regulating c-met Expression in B16-LS9 Cells

Previous evidence indicated that c-met expression in B16 melanoma cells could be induced by MSH treatment [Rusciano et al., 1999]. Results illustrated in Figure 5A clearly show that MSH has the ability to enhance c-met promoter transcription ability, at the point that, after 24 h of treatment, it is comparable to the basal activity observed in B16-LS9 cells. On the other hand, results reported in Figure 5B indicate

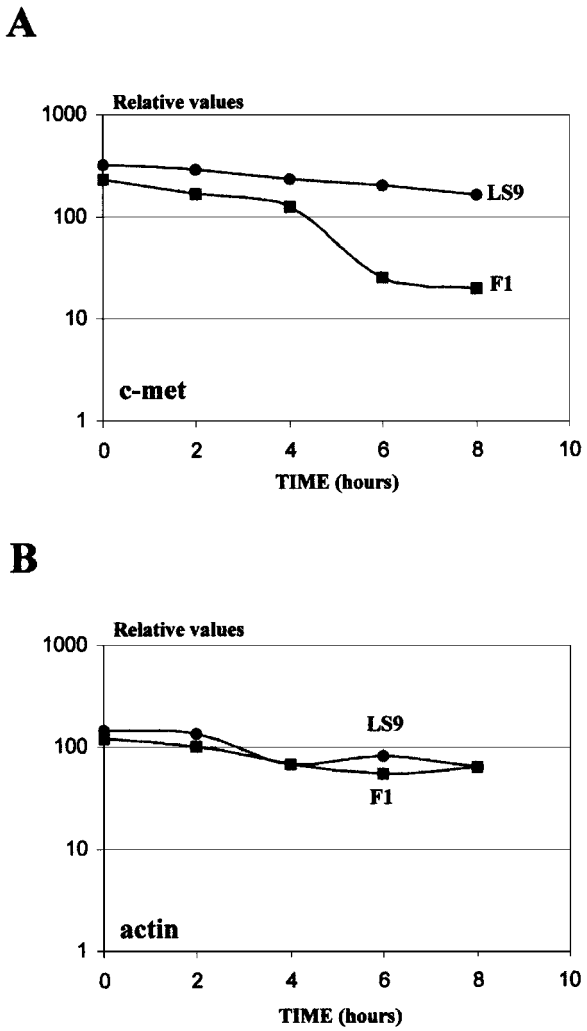

Fig. 4. Messenger RNA stability assay. Poly-A^+ RNAs were prepared at the indicated times from actinomycin-treated B16 cells and analyzed by Northern blot with specific probes for either c-met or β-actin, and the resulting bands visualized by chemiluminescence on an autoradiography film. The densitometric analysis of the bands visible on the film is reported in **A:** for c-met, and in **B:** for actin.

that treatment of c-met-overexpressing B16-LS9 cells with an antagonist of MSH, the agouti peptide, resulted in a decrease of c-met expression, to levels comparable to those observed in B16-F1 cells. This observation raised the intriguing possibility that c-met overexpression in B16-LS9 cells is sustained by an autocrine mechanism, whereby cells produce both the ligand (MSH) and the receptor (MCR-I). To verify this hypothesis, we set out first to see whether agouti could effectively prevent c-met induction by MSH treatment. The experiment was carried out in B16-F1 cells, in which the induction is better evident. Results shown in Figure 6A indicate that while a short-term (8 h) treatment with the agouti peptide alone has no influence on c-met or tyrosinase expression, the simultaneous presence of MSH and agouti results in a complete block of c-met induction by MSH. Next, we carried out long term (48 h) treatments of both B16 cell lines (F1 and LS9) in the continuous presence of the agouti peptide, but without any exogenously added MSH. Results reported in Figure 6B show that there is a progressive decrease in the amount of active (phosphorylated) c-met in the presence of agouti, even though what remains is still higher in B16-LS9 than in B16-F1. The total amount of c-met is drastically decreased in B16-LS9 cells, whereas no changes are apparent in B16-F1 cells. Interestingly, in the presence of agouti, both B16 cell lines express similar amounts of c-met protein, which is, however, more phosphorylated in B16-LS9 cells. This difference could be the consequence of a different cell surface distribution of c-met, since we have shown that its phosphorylation levels depend on its dispersion over the membrane [Rusciano et al., 1996]. Levels of tyrosinase are also decreased in the presence of agouti in both B16 cell lines, although B16-F1 cells seem to be more resistant to this effect.

Finally, Figure 6C shows that cell morphology is also affected by the presence of the agouti peptide. Normally, the majority of B16-LS9 cells in culture display a dendritic shape, indicative of a differentiated phenotype [Rusciano et al., 1998b]. After 2 days of growth in presence of the agouti peptide, a more regular, polygonal shape becomes predominant, as is expected when the effects of MSH are blocked.

DISCUSSION

Two main relevant conclusions emerge from the results reported in this paper: first, both transcriptional and post-transcriptional mechanisms contribute to the overexpression of c-met in B16-LS9 cells; and second, a self-activation of MCR-1 is at least partially responsible for the sustained activity of c-met and tyrosinase promoters in B16-LS9 cells.

Regulation of c-met Gene Expression: Transcriptional Events

Despite its relevance for tumor malignancy, little is known about the mechanisms controlling c-met expression in normal and cancer

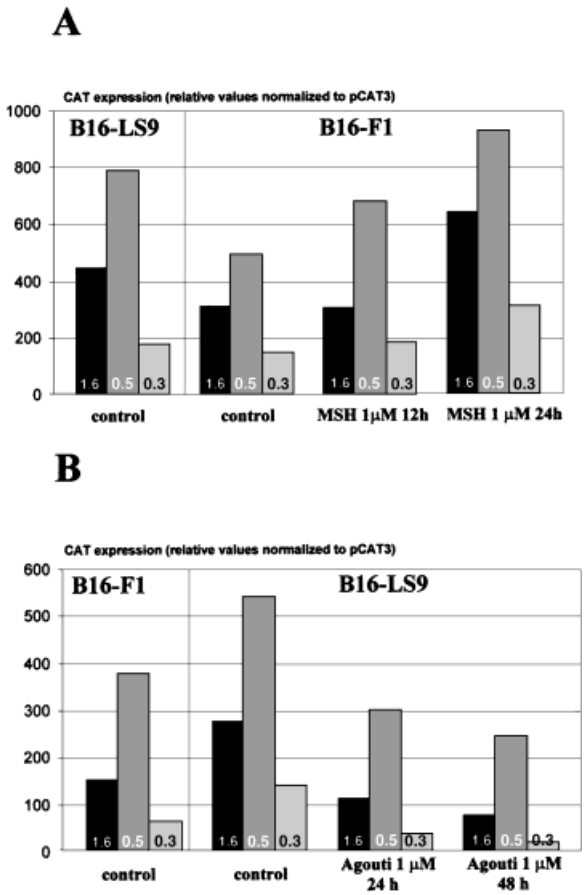

Fig. 5. Modulation of c-met promoter activity by MSH and agouti. B16 melanoma cells were transiently transfected with pCAT plasmids containing the different lengths of c-met promoter, or with control vectors, as in Figure 3. Immediately after transfection, B16-F1 cells were treated with 1 μM MSH for 12 or 24 h (**A**), while B16-LS9 cells were treated with 1 μM agouti for 24 or 48 h (fresh agouti was given every 12 h, to keep a stable active amount of peptide) (**B**). At the end of each treatment time, cell extracts were prepared and analyzed by ELISA for CAT expression. The reported values have been normalized to the pCAT3 values obtained for either cell line at each point, to allow a direct comparison.

cells. Gene amplification of c-met has been reported, however, only in a minority of human colorectal and gastric carcinomas [Di Renzo et al., 1995; Nakajima et al., 1999; Umeki et al., 1999; Wu et al., 1998]. For all the other cancers in which overexpression of c-met has been documented, the regulatory mechanisms remain unknown. The human c-met promoter has been sequenced and analyzed [Gambarotta et al., 1994]. Its pattern of activity suggests the presence of weak negative and positive regulatory elements in the region between 300 and 840 bp upstream to the transcription start site. The region encompassing the first 300 bp strongly up-regulates the promoter. Within this region, a prominent role has been found for ets transcription factors [Gambarotta et al., 1996], which had previously been associated with the generation of an invasive and metastatic behavior [Vandenbunder et al., 1994; Wernert et al., 1994], thus matching the phenotype induced by c-met overexpression. However, thus far no evidence has been reported for an involvement of the ets pathway in human malignancies. An ets-1 consensus sequence (GAGGAAGT) is also present within the mouse c-met promoter [Seol and Zarnegar, 1998], but when we measured in a Western immunoblot the amount of ets-1 transcription factor present in B16-F1 and B16-LS9 cells, we did not find any difference (not shown). Moreover, MSH treatment decreased ets-1 expression in both B16 cells lines, further suggesting that this transcription factor is not involved in the different activity of c-met promoter in these two cell lines. As MSH can induce transcription of both tyrosinase [Englaro et al., 1995] and c-met [Rusciano et al., 1999]; (Fig. 5A), it is likely that common regulatory elements shared by c-met and tyrosinase promoters could be involved in the response of B16 melanoma cells to MSH. A recent report implicated AP-1 and Sp-1 as potent regulators of mouse c-met transcription [Seol et al., 2000]. AP-1 is also activated following MAP kinase activation during cAMP-induced melanogenesis in B16 melanoma cells [Englaro et al., 1995]. Both PKA and MAP kinase activation also regulate c-met expression in B16 cells [Rusciano et al., 1998b, 1999], thus suggesting that AP-1 could be involved in the higher activity of c-met promoter in B16-LS9 cells. Experiments are under way to examine the respective roles of AP-1 and Sp-1 in B16-LS9 cells, by using specific inhibitors of either factor [Seol et al., 2000]. AP2 is also an interesting candidate, because it appears to mediate transcriptional activation in response to two different signal-transduction pathways, one involving PKC, and the other involving PKA [Imagawa et al., 1987], and we have shown that both PKC and PKA are required for c-met induction by MSH treatment [Rusciano et al., 1999]. Moreover, AP2 has been shown to work as a negative regulator of growth in cancer cells [Zeng et al., 1997], and one characteristic of B16-LS9 is indeed a low growth rate, both in vitro and in vivo [Rusciano et al., 1993]. Other possible candidates are Oct-1 and GATA-1, for which

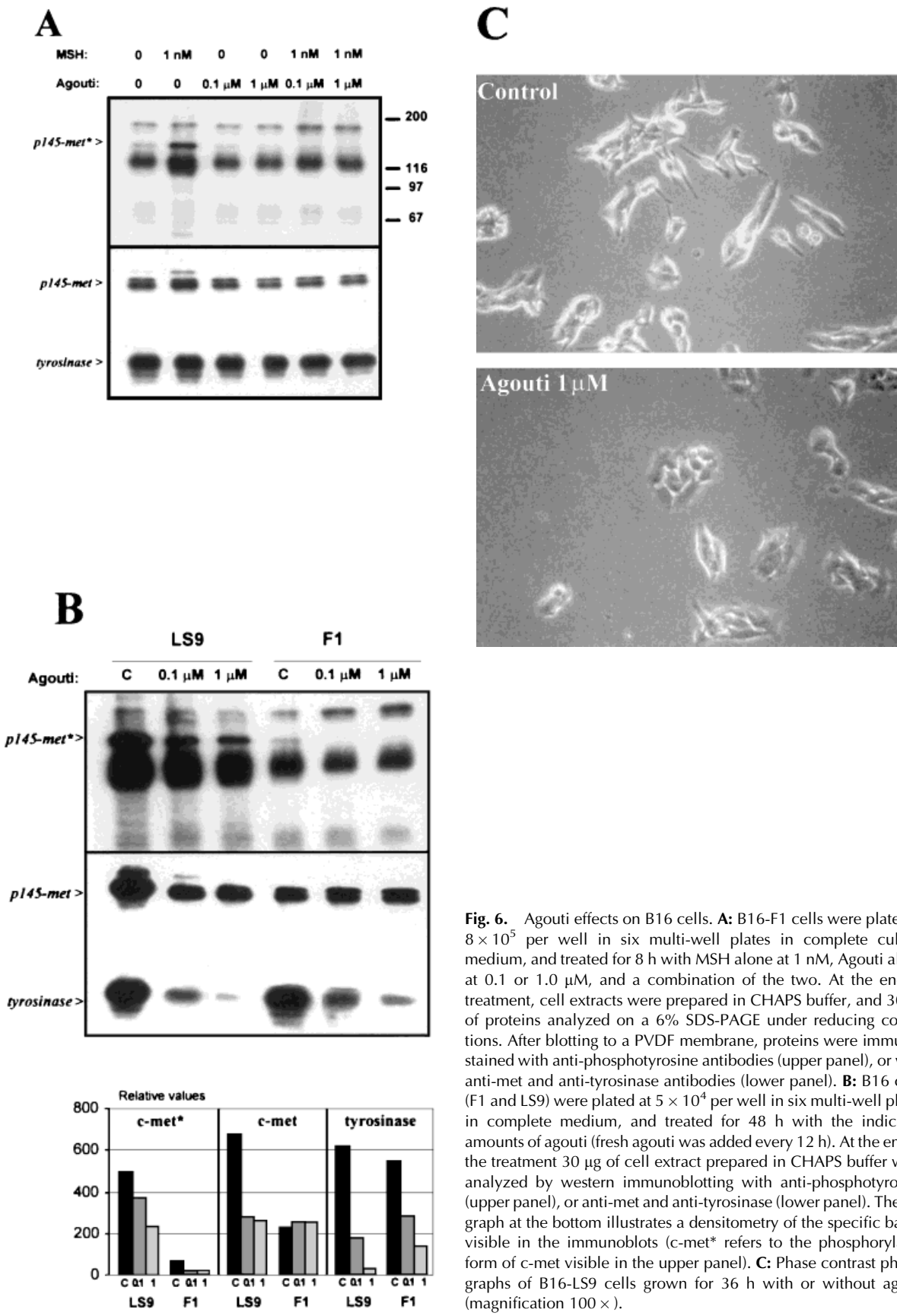

Fig. 6. Agouti effects on B16 cells. **A:** B16-F1 cells were plated at 8×10^5 per well in six multi-well plates in complete culture medium, and treated for 8 h with MSH alone at 1 nM, Agouti alone at 0.1 or 1.0 μM, and a combination of the two. At the end of treatment, cell extracts were prepared in CHAPS buffer, and 30 μg of proteins analyzed on a 6% SDS-PAGE under reducing conditions. After blotting to a PVDF membrane, proteins were immunostained with anti-phosphotyrosine antibodies (upper panel), or with anti-met and anti-tyrosinase antibodies (lower panel). **B:** B16 cells (F1 and LS9) were plated at 5×10^4 per well in six multi-well plates in complete medium, and treated for 48 h with the indicated amounts of agouti (fresh agouti was added every 12 h). At the end of the treatment 30 μg of cell extract prepared in CHAPS buffer were analyzed by western immunoblotting with anti-phosphotyrosine (upper panel), or anti-met and anti-tyrosinase (lower panel). The bar graph at the bottom illustrates a densitometry of the specific bands visible in the immunoblots (c-met* refers to the phosphorylated form of c-met visible in the upper panel). **C:** Phase contrast photographs of B16-LS9 cells grown for 36 h with or without agouti (magnification 100 ×).

the respective consensus sequences are present both in tyrosinase and c-met promoters.

Regulation of c-met Gene Expression: Post-Transcriptional Events

Transcriptional activation, though, is not the only mechanism behind c-met overexpression in B16-LS9 cells, and for the first time we also describe a relevant influence of mRNA stability (Fig. 4). Not much is known from the literature about the relative stability of the message for c-met. A fast decay (half life of about 30 min) of c-met transcript has been reported in a human endometrial carcinoma cell line [Moghul et al., 1994], and no instances have been reported to our knowledge, in which the stability of the transcript contributes to the increased expression of the receptor. In cases where both transcription efficiency and mRNA stability were considered, the enhancement of c-met expression (due to either interferon-gamma, or to cortisol treatment) was found to depend only on transcription [Blanquaert et al., 2000; Nagahori et al., 1999]. It is not clear yet whether the increased stability of c-met mRNA in B16-LS9 cells is due to modifications at the 3′ end of the message, to a change in the relative abundance of specific mRNA binding proteins, or to a different translational activity of the message. It is interesting, however, to note that during liver regeneration (a process in which the paracrine interaction between HGF/SF and c-met has a pivotal role) [Eguchi et al., 1997; Michalopoulos and DeFrances, 1997]: genes that are transcriptionally activated also exhibit post-transcriptional control at the level of transcript stability [Kren and Steer, 1996], indicating that, under certain circumstances, c-met expression can be regulated in both ways.

Regulation of c-met Gene Expression: Autocrine Induction

The finding that MSH is a potent inducer of c-met gene expression [Rusciano et al., 1999], acting at the level of promoter activation (Fig. 5A), coupled with the knowledge that B16 cells can express both MSH and its receptor (MCR-1) [Lunec et al., 1990; Zubair et al., 1992], suggested the possibility that an autocrine mechanism in B16-LS9 melanoma cells may sustain elevated expression levels of both tyrosinase and c-met. In support of this idea, we found evidence that treatment of B16 cells with the agouti peptide (a potent antagonist of MSH) not only prevented in B16-F1 cells the induction of c-met by MSH (Fig. 6A), but also resulted in B16-LS9 cells in a decrease of c-met promoter activity (Fig. 5B), and a reduced expression of c-met and tyrosinase (Fig. 6B, C). On the contrary, the lesser sensitivity of B16-F1 cells to the effects of agouti, suggests that their baseline levels of c-met is maintained by mechanisms at least partially independent from the activity of MCR-1. However, when we evaluated the amounts of MSH and MCR-1 expressed by the two B16 lines, we could not find any obvious difference that could support the hypothesis of a classical autocrine loop more active in B16-LS9 than in B16-F1 cells (not shown). There are, though, two alternative explanations to these findings. A first possibility is that an intracrine mechanism is responsible for the upregulation of c-met and tyrosinase in B16-LS9 cells. Intracrine mechanisms have been described for growth factors and hormones [Delrieu, 2000; Re, 1999], but not yet for melanocortins. So, if proved to be real, this would be the first case involving this type of receptors. The other possibility is that B16-LS9 cells express a mutated form of the MCR-1, which is constitutively active. Such mutations have been described, and reported as being able to significantly elevate adenyl-cyclase activity in the absence of ligand [Robbins et al., 1993; Cone et al., 1996; Vage et al., 1997]. Preliminary data show indeed that B16-LS9 cells contain higher amounts of cAMP than parental B16-F1 (not shown). In either case, the presence of agouti could be expected to decrease signaling through the receptor. In fact, it has recently been shown that agouti can also function as an inverse agonist of MSH, so that its binding to MCR-1 would transduce an opposite signal to the one conveyed by binding of MSH [Siegrist et al., 1997; Ollmann et al., 1998]. We are now in the process of cloning by PCR the MCR-1 gene from B16-F1 and -LS9 cells in order to compare their sequences and see whether there are changes that might suggest a difference in activity.

In conclusion, results here reported show that:

- c-met overexpression in liver-specific B16-LS9 cells depends on both increased promoter activity and higher transcript stability.
- MSH and agouti have competing effects on c-met promoter activity in B16 cells, thus

resulting respectively either in up- or down-regulation of c-met expression.

- A mechanism based on MCR-1 activation sustains high tyrosinase and c-met expression in B16-LS9 cells. It is not clear yet, however, whether an intracrine mechanism, or an activating mutation of the melanocortin receptor could also be involved in the regulation of c-met and tyrosinase expression in B16-LS9 cells.

Experiments are now under way to establish the nature of the transcription factor(s) involved in the up-regulation of c-met expression in B16-LS9 cells to verify their compatibility with a mechanism that involves an increased activity of the melanocortin receptor.

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