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Cloning of a Novel Malignant Melanoma-derived Growth-Regulatory Protein, MIA[1]

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ABSTRACT

Growth and progression of malignant melanoma cells is influenced by a complex network of growth-stimulating and -inhibiting factors produced by both the tumor cells and the local environment. Here we report the purification and molecular cloning of a novel growth regulating protein, designated melanoma inhibitory activity (MIA) and provide a preliminary functional characterization. MIA is translated as a 131-amino acid precursor and processed into a mature 107-amino acid protein after cleavage of a putative secretion signal. A murine complementary DNA was isolated that encoded a MIA-protein with 88% amino acid identity. MIA is secreted into the culture supernatant by several malignant melanoma cell lines as an M_r 11,000 autocrine growth factor and acts as a potent tumor cell growth inhibitor for malignant melanoma cells and some other neuroectodermal tumors, including gliomas. MIA has no homology to any other known protein and, therefore, represents a novel type of growth-regulatory factor. Furthermore, we describe a molecular approach to express functionally active MIA in *Escherichia coli*, which might be attractive as a future antitumor therapeutical substance.

INTRODUCTION

Tumor growth and proliferation is regulated by a complex network of different growth factors, which play critical roles in tumorigenesis, invasion, and tumor progression. Growth-regulatory factors are produced by either the environment or by the tumor and may control cellular functions in an autocrine and paracrine manner. Melanoma cells have been shown to produce TGF[7]-α, TGF-β1, TGF-β2, TGF-β3, PDGF-A, PDGF-B, melanoma growth stimulating activity (MGSA), bFGF, IL-1α and IL-1β, IL-6, IL-8, and tumor necrosis factor α (1, 2); however, malignant progression of melanoma cells is accompanied by a successive decline in exogenous growth factor requirements (3). The purification and molecular cloning of these factors have provided new insights into tumor pathophysiology and, possibly, new targets for antitumor therapy.

Whereas most melanomas that have progressed to widespread metastatic disease relapse very soon after therapeutic intervention, rare cases of spontaneous, prolonged tumor-free survival have been reported (reviewed in Ref. 4). A molecular understanding of tumor growth regulation in these cases might reveal key regulatory factors with general impact on cell growth control and tumor progression. Therefore, we studied a tumor cell line, HTZ-19, which was derived from a human melanoma central nervous system metastasis of a patient with prolonged tumor-free intervals. HTZ-19 cells, cultured under serum-free conditions, produced melanin and were shown to express bFGF, PDGF-A, TGF-β_1, and TGF-β_2, as well as several other, potentially tumor progression associated-proteins, including diazepam-binding inhibitor and tissue inhibitor of metalloproteinases-2 (5–7). Although HTZ-19 cells secrete a number of positive autocrine growth-regulatory proteins, we identified a new potent MIA from supernatants of this cell line that might explain its exceptionally slow tumor progression *in vivo* (8).

We now report the molecular cloning of this novel growth-regulatory protein and compare its sequence obtained from human and mouse cDNA libraries. Malignant melanoma is the neoplasm with the largest increase in incidence over the past years (9). A growth-inhibitory protein like MIA may, therefore, play a significant role in cancer therapy. For this approach, we have expressed and purified MIA using both prokaryotic and eukaryotic expression vectors and provided a preliminary functional characterization of the recombinant protein.

MATERIALS AND METHODS

Cell Lines, Tissue Culture, and Proliferation Assays. All melanoma cell lines were grown in Ham's F-12/Dulbecco's modified Eagle's medium supplemented with 10% FCS as monolayers under standard culture conditions, as described (8). HTZ-1014, HTZ-1004, HTZ-320, and HTZ-318 were established from human malignant melanoma central nervous system tumor metastases; G-361 (CRL 1424) and SK-Mel 3 (HTB-69) were obtained from American Type Culture Collection, and Mel Im was a gift from R. Riethmüller and J. Johnson, Munich, Germany. HTZ-19 cells were grown in serum-free medium. H-36 human fibroblasts were grown from normal human skin biopsies and were kindly provided by the Department of Human Genetics, University of Würzburg,, Würzburg, Germany. The glioma cell lines HTZ-17, HTZ-146, HTZ-298 (glioblastoma multiforme), HTZ-262, and HTZ-243 (anaplastic astrocytoma, WHO grade III) were established from human tumor biopsies and grown in Dulbecco's modified Eagle's medium-10% fetal calf serum as standard monolayer cultures (6, 10). PA-1 human teratocarcinoma cells were obtained from the American Type Culture Collection (CRL 1572) and were grown in minimal essential medium-10% FCS. The subclone 9117 used in this study and a detailed transfection protocol using calcium phosphate precipitation have been described (11). Peripheral blood mononuclear cells were separated by Ficoll-Hypaque gradient centrifugation of venous blood received from healthy donors (12).

[^{3}H]Thymidine incorporation was measured as described (8). For growth curves, 2×10^4 cells were seeded into 24-well dishes (Nunc, Roskilde, Denmark), and the number of viable cells was counted daily. Duplicates were treated in parallel with medium supplemented with recombinant MIA or control purificate (see below). The viability of cells was confirmed by trypan blue exclusion. For dose-response curves, cells were counted only on day 5.

Purification of MIA from HTZ-19 Supernatants. A detailed protocol for the recovery of the HTZ-19 conditioned tissue culture supernatant and purification of MIA has been described previously (7, 8). Briefly, 5 liters of supernatants were concentrated by membrane ultrafiltration (YM-2 membrane cutoff, M_r 2,000; Amicon, Danvers, MA), and the retentate was dialysed against 0.1 M acetic acid. The protein concentrate was cleared by ultracentrifugation (28,000 rpm; Ti 60 rotor; Beckmann, Munich, Federal Republic of

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[7] The abbreviations used are: TGF, transforming-growth factor; PDGF, platelet-derived growth factor; bFGF, basic fibroblast growth factor; IL, interleukin; DHFR, dihydrofolate reductase; rpHPLC, reverse-phase high performance liquid chromatography; MIA, melanoma inhibitory activity; FCS, fetal calf serum; TFA, trifluoroacetic acid; cDNA, complementary DNA; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

Germany) and separated by BioGel P-10 (Bio-Rad, Richmond, CA) gel permeation chromatography in 1 M acetic acid. Fractions 39–45, containing the highest specific biological activity as determined by [^{3}H]thymidine incorporation, were pooled (P-10 pool) and lyophilized again. After solubilization in 0.1% TFA, proteins were further separated by rpHPLC on a MinoRPC column (Pharmacia, Freiburg, Federal Republic of Germany). Proteins were eluted with a three-step gradient: 2–25% solvent B within 5 min, 25–50% solvent B within 120 min, and 50–100% within 5 min. Solvent A was 0.1% TFA, and solvent B was 80% acetonitrile-0.056% TFA. Gradient was started 5 min after probe injection (100 μg P-10 pool).

Protein Sequence Analysis. The rpHPLC fraction 42 exhibited the highest biological activity and was used for sequence analysis after drying under vacuum. Protein sequencing was performed on an Applied Biosystems 477 A gas-phase sequencer (Boulder, CO) that was equipped with a 120-APTH analyzer. After determination of the NH_2-terminal sequence, the protein was dissolved in 8 M urea-0.4 M NH_4HCO_3 (pH 7.9) and 5 μl-45 mM dithiothreitol and incubated for 15 min at 50°C. After the addition of 5 μl-100 mM iodacetamide and incubation for 15 min at room temperature, either trypsin or Asp-N proteases were added in 140 μl H_2O (0.003 units of enzyme/mg protein), and digestions were performed for 24 h at 37°C. The resulting peptides were separated on a Vydac C_{18} 2.1 x 250 mm rpHPLC column by a gradient of buffer A (0.06% TFA in H_2O) and buffer B (0.052% TFA-80% acetonitrile). Three gradient steps were performed: 2–40% buffer B within 60 min, 40–75% buffer B within 30 min, and 75–98% buffer B within 15 min at a flow rate of 0.2 ml/min. Finally, the peptides were subjected to NH_2-terminal sequencing.

Isolation of Recombinant cDNA Clones. For reverse transcriptase-polymerase chain reaction amplification of a partial MIA cDNA, the primers UP-1 (5′-TGT GAA TTC AAG TTI A/TC/GI GCI GAC/T CAA/G GAA/G TG- 3′) and DP-1 (5′TGT GTC GAC TGT TCG TAG AAA/G TCC CAC/T TTG/A TC-3′) were used matching amino acid residues 34–41 and 131–124 of the MIA protein, as predicted from microsequenced peptides. Restriction sites *Eco*RI and *Sal*I, added for convenient subcloning, are underlined. First-strand cDNA was synthesized with murine Moloney leukemia virus-reverse transcriptase using 0.5 μg DP-1 and 10 μg HTZ-19 RNA, according to a protocol that was described previously (13), and cDNA was amplified by 32 cycles using the following temperature profile: 1 min at 95°C, 1 min at 55°C, and 0.5 min at 72°C. The amplified product was subcloned into the vector pBluescript (Stratagene, La Jolla, CA) and verified by sequencing as a partial MIA cDNA clone that encoded all of the 85 amino acids obtained from peptide sequencing. The insert was then used as a radiolabeled probe (14) to screen 300,000 recombinant plaques from a HTZ-19 cDNA library in the phage λgt11. Handling of phages, plaque lifting, screening, and Southern hybridizations were performed according to standard protocols (15). Ten positive and independently derived plaques were purified; then the inserts were subcloned into the plasmid pBluescript sequenced on both strands. The longest cDNA insert, huMIA-7, (Fig. 2*c*) was used to screen 300,000 plaques from a commercially available murine embryo cDNA library (Novagen, Madison, WI; embryo day 13.5), resulting in 5 recombinant clones with overlapping cDNA inserts. The inserts were recovered using CRE-loxP-mediated plasmid excision and fully sequenced on both strands. Sequencing was performed in part according to the standard Sanger protocol (16) and in part by cycle sequencing using an automatic DNA sequencer (Applied Biosystems).

Northern Blots. RNA was isolated as described (17). Total cellular RNA (20 μg/lane) was loaded on a 1% formaldehyde-agarose gel and transferred to nylon membranes according to a standard protocol (15). The complete huMIA-7 cDNA insert was radioactively labeled (14) and used as a probe. Final washes were performed in 1 × standard saline-citrate (0.15 M NaCl, 0.15 M sodium citrate) for two times for 1 h each at 68°C.

Preparation of Antisera and Western Blots. For preparation of antisera, a peptide corresponding to MIA amino acids 4–18 of the mature peptide was synthesized and coupled via a COOH-terminal cysteine residue to bovine serum albumin using (*m*-maleimidobenzoic acid-*N*-hydroxysuccinimide ester) as a bifunctional reagent (18). Rabbits were immunized by three s.c. injections. Supernatants for Western blotting were prepared as described above. SDS-PAGE and Western transfers to nitrocellulose membranes were performed according to the method of Kyse-Anderson (19). After blocking with ovalbumin, blots were incubated for 2 h with antisera diluted 1:50 in TBST (Tris-buffered saline with 0.05% Tween 20). Washing of blots was followed by incubation with a second alkaline phosphatase-conjugated mouse anti-rabbit antibody. For color development, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate was used.

Expression and Purification of Recombinant Protein. *Escherichia coli* M15(pREP4) cells carrying the DHFR-MIA fusion protein expression plasmid pQE 40-MIA were grown to an absorbance of OD_{600} = 0.6 and induced by 1 mM isopropyl-1-thio-β-D-galactopyranoside for 4 h and lysed by sonication. The soluble protein fraction was subjected to nickel-ion metal chromatography as described in detail elsewhere (13). The fused DHFR-MIA proteins, linked by a specific IgA protease recognition sequence S-R-P-P-S-P, were cleaved by incubation of the ion metal affinity chromatography column with 0.1 μg/ml IgA protease (Boehringer Mannheim, Mannheim, Federal Republic of Germany) for 10 h at 37°C. Finally, the cleaved MIA protein was eluted into PBS (pH 7.35). In parallel, the parental pQE 40 vector without a MIA insert was processed identically, including the IgA protease step; the PBS eluate was used as a control for treatment of melanoma cells in tissue culture experiments.

For eukaryotic expression, the huMIA-7 cDNA was ligated into the *Eco*RI site of the vector pCMX pL1 (20) and transiently transfected into PA-1 sc9117 cells using the calcium phosphate precipitation method (13). After the transfection, cells were washed three times in PBS and incubated for an additional 48 h in serum-free minimum essential medium. The supernatant was collected and cleared from cellular debris by centrifugation (10 min at 2500 × *g*). As a control, the empty pCMX-pL1 vector was transfected and processed precisely as the MIA-expression construct.

RESULTS

Purification of MIA from HTZ-19 Supernatants. Purification of MIA from serum-free supernatants of HTZ-19 cells is described in detail elsewhere (7, 8). Fractions from Biogel P-10 gel column chromatography with the highest specific inhibitory activity were pooled (designated P-10 pool) and separated further by rpHPLC. The main activity was eluted from the rpHPLC column in fraction 42 and shown to contain a single protein with a M_r 11,000. This protein was designated MIA. Growth inhibition was accompanied by a change in cytomorphology, resulting in a compact, rounded-up and less adherent phenotype (Fig. 1). However, exposure to MIA is not cytotoxic, as demonstrated by trypan blue exclusion and reversal of growth inhibition after removal of MIA (data not shown).

Molecular Cloning of MIA (8). After purification and preliminary functional characterization of the M_r 11,000 MIA protein, direct NH_2-terminal sequencing was performed. To obtain internal peptide fragments, the protein was reduced, carboxymethylated, and digested with trypsin and Asp-N-protease, respectively. Finally, fragments were separated by rpHPLC and microsequenced by Edman degradation. Ten partially overlapping peptide sequences were obtained covering a total of 89 amino acids, as shown in Fig. 2*a*. Two degenerated oligonucleotides were synthesized, matching predicted amino acid codons from the NH_2-terminal and COOH-terminal peptides (Fig. 2*a*). Using these primers, a cDNA fragment was amplified by reverse transcriptase-polymerase chain reaction from HTZ-19 RNA. This fragment served as a probe to screen 3 × 10^5 plaques of a λgt11 cDNA library from the HTZ-19 cell line, resulting in 10 independent overlapping cDNA clones. Two of these clones covered the entire open reading frame [Fig. 2, *a* (top) and *c*]. We further used the longest human cDNA clone huMIA-7 to screen 3 × 10^5 plaques of a commercially available murine embryo cDNA library (day 13, Ref. 5; Novagene) resulting in five overlapping cDNA clones, two of which covered the complete murine open reading frame (Fig. 2*a*, *bottom*).

The human and murine protein sequences are aligned in Fig. 2*a*. A search by Fasta through the EMBL data base (peptide search wordsize, 2; sequence search wordsize, 6; Ref. 21) yielded no significant homology to any other known protein or nucleic acid sequence. A comparison between the open reading frame predicted from the human cDNA and the sequence information obtained from the NH_2-terminal peptide sequence of the originally purified M_r 11,000 protein

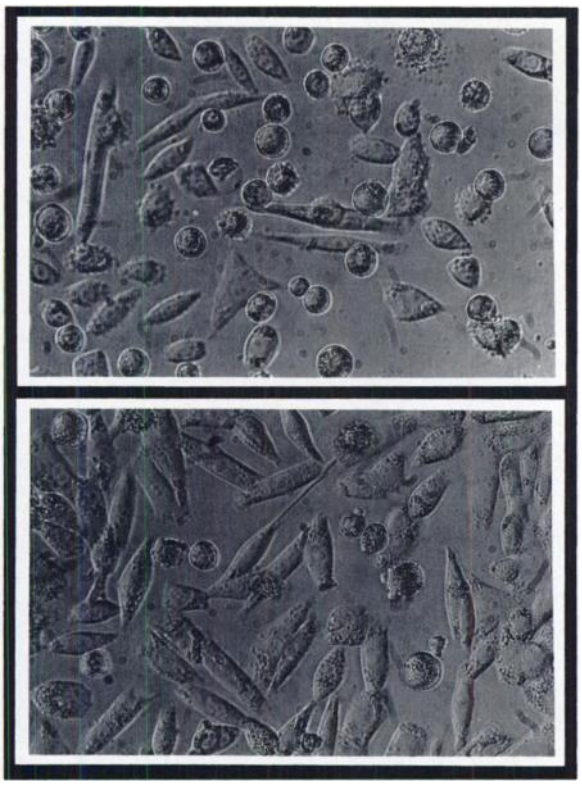


Fig. 1. Morphology of HTZ-19 cells treated with purified MIA. Cells treated with HPLC-purified MIA (*A*) in comparison to untreated cells (*B*) 4 h after start of treatment. MIA-treated cells are beginning to roundup.

revealed that human MIA is translated as a 131-amino acid precursor molecule that is processed into a mature 107-amino acid protein after cleavage of a 24-amino acid secretion signal. The site of proteolytic cleavage is indicated by an *arrow* in Fig. 2*a*. These 24 amino acids are extremely hydrophobic and are cleaved after the amino acid residues Arg-Gly, features that are well known from other eukaryotic secretion signals (22). The murine secretion signal is slightly shorter than the human sequence and differs in 10 of 23 residues. However, the key features are well conserved, *i.e.*, hydrophobicity and a strongly charged arginine at the second-last residue.

A hydrophobicity profile using the Kyte-Doolittle algorithm revealed four hydrophobic domains in addition to the hydrophobic secretion signal (Fig. 2*b*). Although the mature murine peptide sequence differs in 13 of 107 amino acids, the overall structure of hydrophobic and hydrophilic domains is highly conserved. Inspection of the sequences further revealed four conserved cysteine residues (Fig. 2*a*, *asterisks*), which are likely to be relevant for the correct three-dimensional folding of the protein.

Expression of MIA mRNA and Protein. We then examined the expression of MIA mRNA in various cell lines of different histogenetic origin. A single mRNA of ~750 bases is detected in every melanoma cell line ($n = 8$) analyzed, and in 1 of 5 glioma cell lines (Fig. 2*a*). No message is found in human lymphomononuclear cells ($n = 5$) or in fibroblasts ($n = 2$). Thus, MIA appears to be frequently expressed in neuroectodermal tumor cell lines. In addition, no MIA mRNA expression in normal tissues was detected using a mouse multitissue Northern blot (data not shown).

A polyclonal rabbit antiserum, produced by repeated immunization with a MIA-specific NH_2-terminal 15-mer peptide coupled to bovine serum albumin, recognizes MIA protein throughout all purification steps of HTZ-19 tissue culture supernatants. MIA protein-related immunoreactivity is also detected in supernatants of two other melanomas (HTZ-318 and HTZ-320) and, to a lesser extent, in one glioma cell line (HTZ-243; Fig. 3*b*). Unfortunately, neither the antiserum nor the purified immunoglobulin fraction neutralized the growth inhibitory effect of MIA.

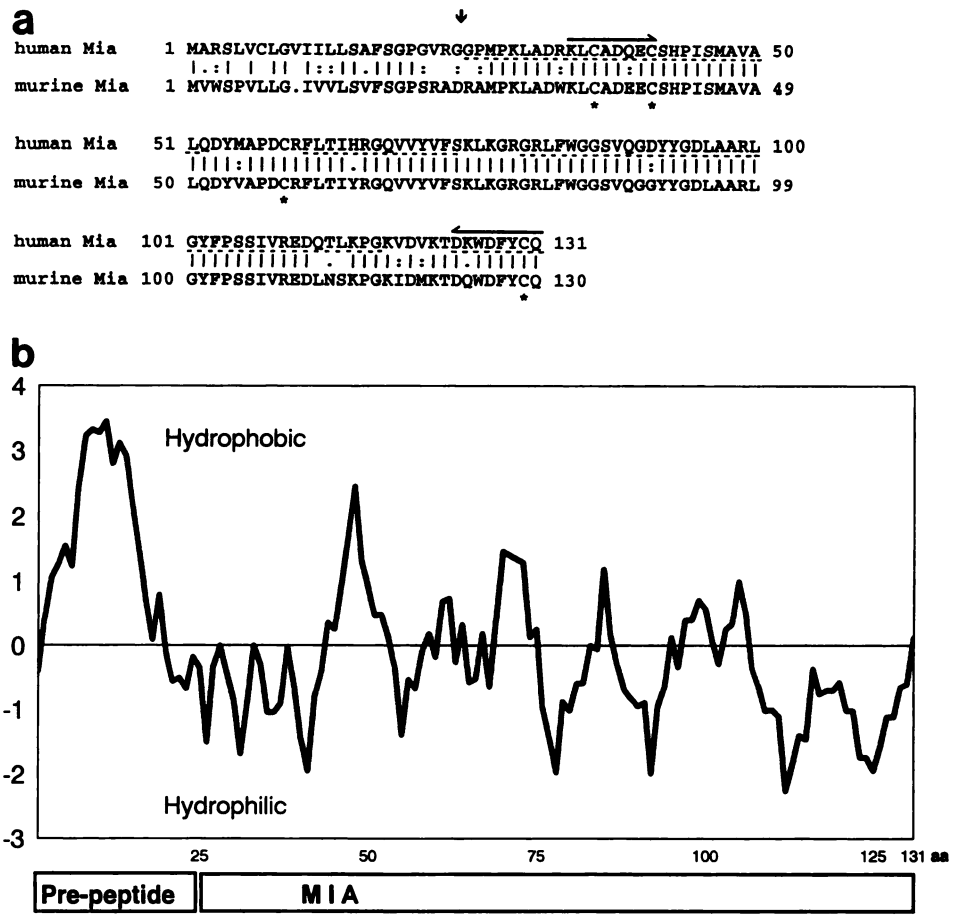
Expression of Recombinant MIA Protein. We next aimed to prove that the cloned gene codes for a protein with the identical biological activity as the originally purified MIA protein. Therefore, we cloned the MIA cDNA into the prokaryotic expression vector pQE 40 (Quiagen, Chatsworth, CA). To find out whether posttranslational modifications are required for biological activity, we also constructed a eukaryotic vector using the cytomegalovirus expression plasmid pCMX-pL1 (20). Recombinant bacterial MIA was expressed as a fusion protein with histidine-tagged DHFR (23) and purified by ion metal affinity chromatography. The fusion protein was expressed abundantly in *E. coli*, mostly as inclusion bodies. However, a fraction of the protein was soluble and could be cleaved while attached to the chromatography column using the IgA protease. Finally a single M_r 11,000 MIA protein was eluted from the column at a purity greater than 95%, as verified by silver-stained SDS-PAGE (Fig. 4). For eukaryotic MIA expression, the plasmid pCMXpL1-MIA was transiently expressed in the teratocarcinoma cell line PA-1 (13). PA-1 cells were chosen since they are efficiently transfected by the calcium phosphate technique and express no endogenous MIA-related mRNAs, as verified by Northern hybridizations (data not shown). Serum-free tissue culture supernatants were collected 48 h after transfections and tested for secreted MIA activity. SDS-PAGE analysis revealed that an M_r 11,000 protein is secreted into the tissue culture supernatant by pCMXpL1-MIA transfected PA-1 cells and is absent in control transfections using the empty vector pCMXpL1 (Fig. 4). Western blot analysis revealed that the prokaryotic recombinant MIA reacted with the MIA-specific antiserum (Fig. 3*b*).

Then we tested the biological activity of the recombinant MIA proteins. As shown in Table 1, both the prokaryotic and eukaryotic recombinant MIA inhibited [^{3}H]thymidine incorporation of HTZ-19 melanoma and HTZ-243 glioma cells and stimulated H-36 fibroblasts. No alteration of [^{3}H]thymidine incorporation was observed with HTZ-17 glioblastoma cells. Thus, the effects of recombinant MIA proteins are identical to those observed using the original protein from HTZ-19 supernatants. The prokaryotic MIA protein was approximately as active as the originally purified MIA protein (approximately 50% inhibitory dose: 20–30 ng/ml) and demonstrated a clear dose-response relationship when tested upon Mel Im and HTZ-19 melanoma cells (Fig. 5*a*).

To confirm the data we obtained from [^{3}H]thymidine incorporation, we measured cell growth curves using HTZ-19 cells and, in addition, an independent human melanoma cell line, Mel Im. Duplicate samples were seeded into 6 well dishes and treated with purified recombinant prokaryotic MIA and supernatant containing MIA protein from eukaryotic expression. As controls, cells were treated with equivalent amounts of purified supernatants obtained from empty expresson vectors (see "Materials and Methods"). The effects were quantified as cell growth curves by counting cells daily. As demonstrated in Fig. 5, *b* and *c*, the Mel Im cells are extremely sensitive to MIA and are rapidly growth arrested between days 2 and 3 at a cell number 90% below control cultures. HTZ-19 cells are less sensitive to MIA; however, a significant reduction in cell number, approximately 60%, was found after 7 days.

DISCUSSION

Evidence has accumulated indicating that proliferation and invasion of tumor cells are regulated by a complex network of positive and



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1	CCA	GCA	CCC	CCT	TGC	TCA	CTC	TCT	TGC	TCA	CAG	TCC	ACG	ATG	GCC	CGG
1														Met	Ala	Arg
49	TCC	CTG	GTG	TGC	CTT	GGT	GTC	ATC	ATC	TTG	CTG	TCT	GCC	TTC	TCC	GGA
4	Ser	Leu	Val	Cys	Leu	Gly	Val	Ile	Ile	Leu	Leu	Ser	Ala	Phe	Ser	Gly
97	CCT	GGT	GTC	AGG	GGT	GGT	CCT	ATG	CCC	AAG	CTG	GCT	GAC	CGG	AAG	CTG
20	Pro	Gly	Val	Arg	Gly	Gly	Pro	Met	Pro	Lys	Leu	Ala	Asp	Arg	Lys	Leu
145	TGT	GCG	GAC	CAG	GAG	TGC	AGC	CAC	CCT	ATC	TCC	ATG	GCT	GTG	GCC	CTT
36	Cys	Ala	Asp	Gln	Glu	Cys	Ser	His	Pro	Ile	Ser	Met	Ala	Val	Ala	Leu
193	CAG	GAC	TAC	ATG	GCC	CCC	GAC	TGC	CGA	TTC	CTG	ACC	ATT	CAC	CGG	GGC
52	Gln	Asp	Tyr	Met	Ala	Pro	Asp	Cys	Arg	Phe	Leu	Thr	Ile	His	Arg	Gly
241	CAA	GTG	GTG	TAT	GTC	TTC	TCC	AAG	CTG	AAG	GGC	CGT	GGG	CGG	CTC	TTC
68	Gln	Val	Val	Tyr	Val	Phe	Ser	Lys	Leu	Lys	Gly	Arg	Gly	Arg	Leu	Phe
289	TGG	GGA	GGC	AGC	GTT	CAG	GGA	GAT	TAC	TAT	GGA	GAT	CTG	GCT	GCT	CGC
84	Trp	Gly	Gly	Ser	Val	Gln	Gly	Asp	Tyr	Tyr	Gly	Asp	Leu	Ala	Ala	Arg
337	CTG	GGC	TAT	TTC	CCC	AGT	AGC	ATT	GTC	CGA	GAG	GAC	CAG	ACC	CTG	AAA
100	Leu	Gly	Tyr	Phe	Pro	Ser	Ser	Ile	Val	Arg	Glu	Asp	Gln	Thr	Leu	Lys
385	CCT	GGC	AAA	GTC	GAT	GTG	AAG	ACA	GAC	AAA	TGG	GAT	TTC	TAC	TGC	CAG
116	Pro	Gly	Lys	Val	Asp	Val	Lys	Thr	Asp	Lys	Trp	Asp	Phe	Tyr	Cys	Gln
433	TGA	GCT	CAG	CCT	ACC	GCT	GGC	CCT	GCC							
132	End															

Fig. 2. Molecular cloning of MIA. *A*, comparison of the amino acid sequence of human (*top*) and mouse (*bottom*) MIA. Conserved cysteines are marked by an *asterisk*, sequences used for primer synthesis by *upper linings*. (——), peptide sequences obtained from microsequencing. *Arrow*, cleavage site of signal protease. *B*, Kyte-Doolittle hydrophobicity analysis of the MIA protein (32). A strong hydrophobic secretion signal is followed by additional four hydrophobic domains. *C*, cDNA sequence of human MIA.[8]

negative growth regulatory signal molecules. Extended studies of malignant melanomas have shown that autocrine factors produced by tumor cells of advanced progression include at least MGSA and bFGF, which are not or only faintly expressed in normal melanocytes (1, 24, 25). It was, therefore, intriguing to study growth regulatory proteins secreted by the human melanoma cell line HTZ-19 that were derived from a tumor with exceptionally slow progression *in vivo* (8). Previous analyses revealed that HTZ-19 cells were stimulated in an autocrine fashion by PDGF and bFGF and, thus, were able to grow independent of exogenous growth factors in serum-free medium (5, 6). We were able to further identify several other proteins secreted by HTZ-19 cells that are implicated in regulation of tumor progression and invasion. Among these factors were TGF-β_1, tissue inhibitor of metalloproteinases (7, 26), diazepam-binding inhibitor (27), and a fourth, thus far uncharacterized, protein MIA (8), conferring growth inhibition upon melanoma cells. These data indicated that both tumor

[8] The cDNA sequence has been submitted to the EMBL Data Library under the accession number X 75450.

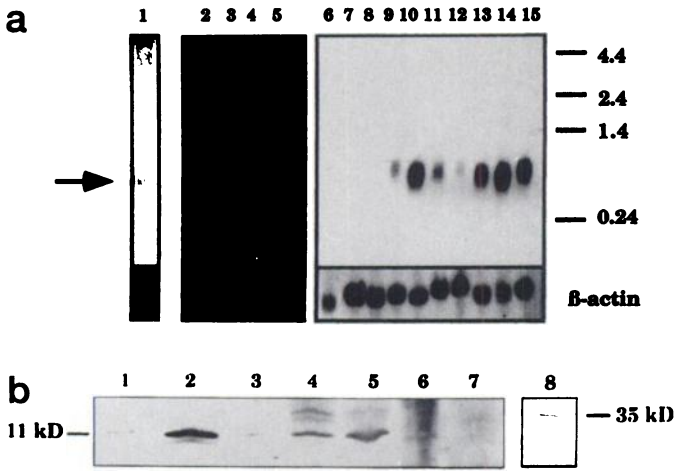

Fig. 3. Expression pattern of MIA mRNA and protein. *A*, Northern blot analysis of MIA mRNA expression in the cell line Mel Im (*Lane 1*; human malignant melanoma); peripheral blood mononuclear cells (*Lane 2*); HTZ-298 (*Lane 3*) and HTZ-262 (*Lane 4*) (glioblastoma multiforme); HTZ-243 (*Lane 5*; human malignant astrocytoma, WHO grade III); H-36 (*Lane 6*; normal human skin fibroblasts); HTZ-17 (*Lane 7*) and HTZ-146 (*Lane 8*) (glioblastoma multiforme); HTZ-1014 (*Lane 9*); HTZ-1004 (*Lane 10*); G-361 (*Lane 11*); SK-Mel 3 (*Lane 12*); HTZ-320 (*Lane 13*); HTZ-318 (*Lane 14*); and HTZ-19 (*Lane 15*) (human malignant melanomas). (→), MIA signal . Actin controls are displayed below. *B*, Western Blots using polyclonal anti-MIA-peptide specific antiserum: rpHPLC-purified MIA (*Lane 1*), P-10 pool (*Lane 2*), HTZ-19 (*Lane 3*), HTZ-320 (*Lane 4*), HTZ-318 (*Lane 5*), HTZ-243 (*Lane 6*), and HTZ-17 (*Lane 7*) supernatants and prokaryotic expressed MIA fusion protein (*Lane 8*).

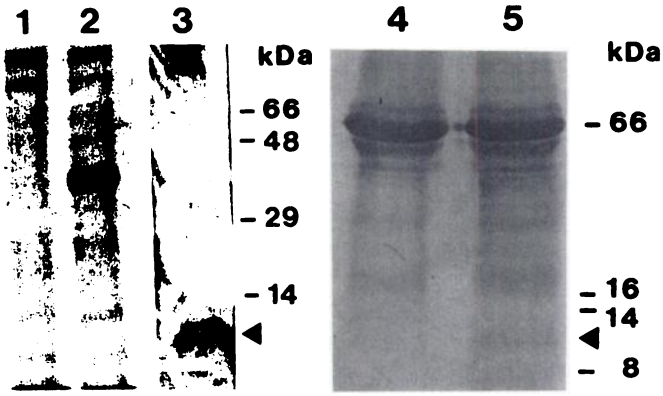

Fig. 4. Recombinant expression of MIA in prokaryotic and eukaryotic systems. SDS-PAGE, 15%, silver stained, showing expression of human MIA in *E. coli* as DHFR-fusion protein, using the vector pQE-40 before (*Lane 1*) and after (*Lane 2*) IPTG induction, as well as affinity-purified MIA after cleavage with IgA protease (*Lane 3*). Eukaryotic expression: supernatants of cells transfected with the empty vector pCMX-pL1 as control (*Lane 4*) and the vector containing the complete MIA cDNA sequence (*Lane 5*).

growth-promoting effects and negative growth-regulatory mechanisms are relevant in this melanoma model.

Here we report on the molecular cloning of the novel growth-regulatory protein, MIA, which has no homology to other known proteins. MIA seems to be widely expressed in malignant melanomas but not in normal fibroblasts or lymphocytes. We purified MIA as an M_r 11,000 protein secreted by melanoma cells and isolated the corresponding human and murine cDNAs. Prokaryotic and eukaryotic expression strategies are described substantiating the function of MIA as a potent negative growth factor and as a potential future therapeutic agent.

Our studies of MIA expression using Northern and Western blot analyses revealed that MIA is widely expressed at variable levels in malignant melanoma cells. We found MIA mRNA in every melanoma cell line tested thus far. Interestingly, we also observed MIA expression in one glioma cell line (HTZ-243; Refs. 28 and 29) raising the possibility that MIA is a more generally expressed neuroectodermal growth regulatory factor. It is intriguing that this MIA-positive cell line HTZ-243 was also derived from a tumor with exceptionally slow clinical progression similar to the melanoma cell line HTZ-19. All cell lines that were sensitive to growth inhibition by MIA also expressed MIA mRNA and exhibited dose-dependent response curves (data not presented). However, there was no direct correlation between the level of MIA expression and the extent of growth inhibition *in vitro*. A number of melanoma cell lines that we analyzed were very sensitive to the effect of MIA, although derived from highly malignant and rapidly progressing tumors. Taken together, these data indicate that cellular signal perception mediated by a receptor and intracellular signal transduction may modulate the effects of MIA. Earlier experiments using fluorescence-activated cell sorter analysis indicated a specific effect on cell cycle regulation. We observed a prolongation of G_1 to S transition, a prolongation of S, and a cell cycle arrest in G_2 (30). This is interesting because MIA differs in this respect from most other growth factors including TGF-β, which arrests the cell cycle at G_1 (31). MIA is not cytotoxic as verified by trypan blue exclusion assays. This observation is rather similar to growth regulatory effects of TGF-β, where direct cytotoxic effects on cells are not observed.

The expression pattern and the physiological role of MIA in normal tissues remain to be determined. We were unable to identify significant levels of MIA mRNA on a Northern blot of 16 different normal murine tissues, including skin and brain, indicating that MIA is either absent or expressed at very low levels in adult tissues. However, we were able to isolate five independent cDNA clones by screening 3×10^5 plaques of a total mouse embryo (day 13.5) cDNA library. Thus, MIA is expressed at high levels during mid-gestation and may, therefore, have a function in regulation of embryonic cell growth and morphogenesis.

Several lines of evidence indicate that MIA is secreted as a small globular protein stabilized by two intramolecular disulfide bonds; all four cysteine residues are conserved between the human and murine sequence, and the overall structure of hydrophobic and hydropilic domains is identical. Reduction of the disulfide bonds results in a slower electrophoretic mobility (data not presented) and in a highly insoluble protein, probably due to dislocation of the many hydrophobic residues to the protein surface. The latter observation was a serious obstacle for production of recombinant MIA protein in bacteria. Consequently, we expressed MIA as a fusion protein with dihydrofolate reductase and renatured the protein at low concentrations after proteolytic cleavage. Interestingly, when we purified MIA using a COOH-terminal histidine tag that was positioned next to the cysteine at amino acid position 130, we obtained a protein that was totally inactive in growth inhibition assays (data not shown). This observa-

Table 1 *Biological activity of recombinant MIA protein*

Antiproliferative effects of purified and recombinant huMIA protein. Data, percentage mean values of [^{3}H]thymidine incorporation (cpm) of treated cells (MIA_{P10}, MIA_{HPLC}) relative to untreated, or treated cells (recombinant MIA, MIA_{eu}, and MIA_{pro}) compared to control treated cells (see Fig. 5a, b) including SD. Data points are means of triplicates; Student's *t* test was applied.

Cell lines[a]	$rMIA_{eu}$[b]	$rMIA_{pro}$[c]	MIA_{HPLC}	MIA_{P10}
HTZ-19 (MM)	26.7 ± 3[d]	12 ± 4[d]	11 ± 8[d]	50 ± 4[d]
HTZ-243 (MG)	40.0 ± 3[e]	77 ± 13[f]	70 ± 14[f]	85 ± 8
HTZ-17 (MG)	93 ± 5	94 ± 9	n.d.	108 ± 2
H-36 (FB)	154 ± 12[g]	164 ± 18[g]	88 ± 21	132 ± 26[h]

[a] MM, malignant melanoma; MG, malignant glioma; FB, human fibroblasts.
[b] $rMIA_{eu}$, recombinant MIA.
[c] $rMIA_{pro}$, recombin MIA protein.
[d] Significant inhibition ($P < 0.001$) compared to individual controls.
[e] Significant inhibition ($P < 0.01$) compared to individual controls.
[f] Significant inhibition ($P < 0.05$) compared to individual controls.
[g] Significant stimulation ($P < 0.01$) compared to individual controls.
[h] Significant stimulation ($P < 0.05$) compared to individual controls.

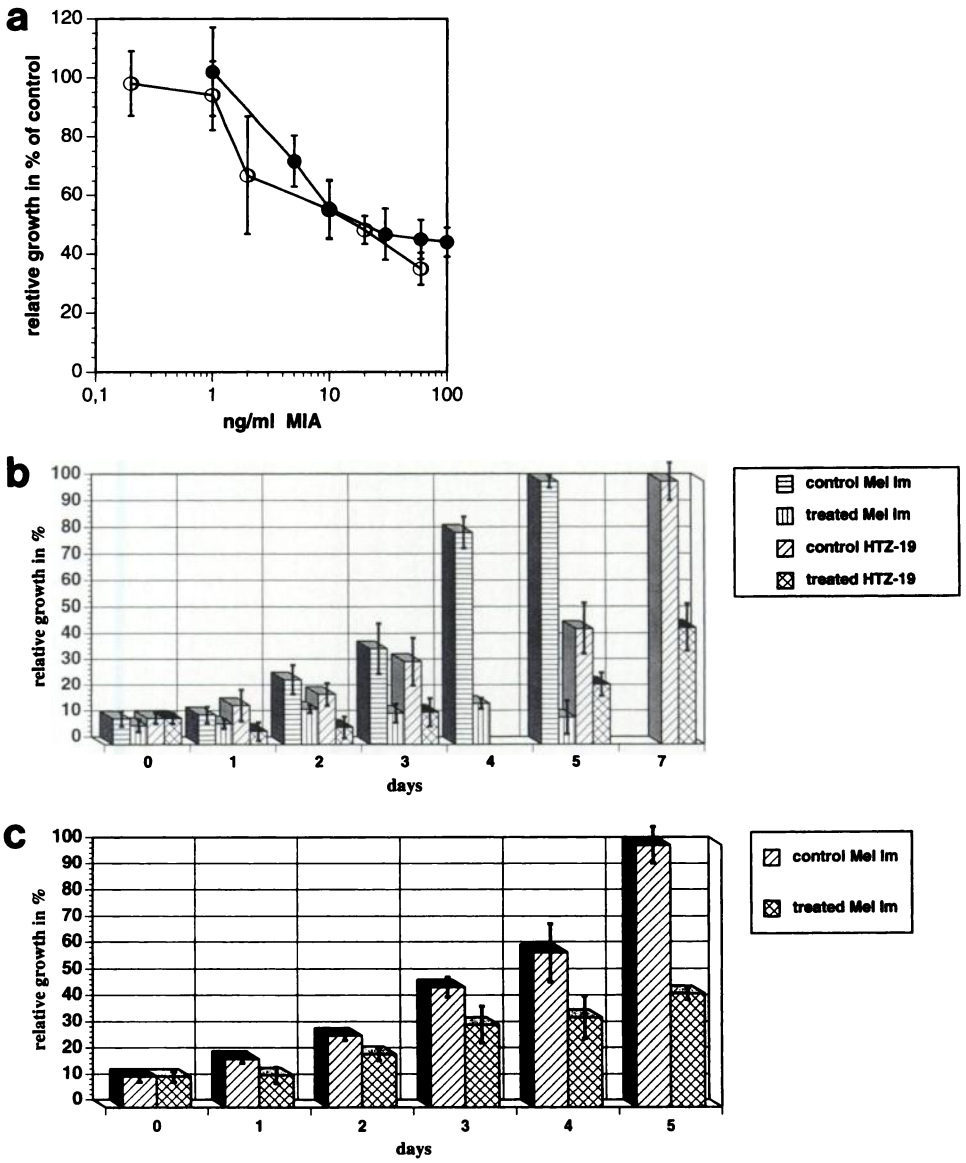

Fig. 5. Biological activity of recombinant MIA protein. *A*, dose-response curves of the melanoma cell lines HTZ-19 (●) and Mel Im (○. Cells were treated for 5 days with the indicated doses of prokaryotic expressed MIA; relative growth was determined by counting cells treated with recombinant MIA compared to controls treated with identically purified protein from *E. coli* bearing the empty vector pQE-40. Protein concentrations were estimated by comparing the intensity of silver staining of MIA with standard proteins in SDS-gels. *B*, cell growth curves of HTZ-19 and Mel Im after treatment with recombinant huMIA, expressed in *E. coli*. Controls were treated with identically purified protein from *E. coli* bearing the empty vector pQE-40 (relative growth is given in a percentage ± SD). *C*, cell growth curve of Mel Im after treatment with supernatatants containing eukaryotic expressed rekombinant huMIA. Controls were treated with supernatants of the PA-1 cells transfected with the empty vector (relative growth is given in percentage ± SD).

tion further points to an important role of the intramolecular cysteine bonds in correct protein folding and function.

Our bacterial expression strategy yielded MIA protein with a purity greater than 95%, as assessed by silver-stained polyacrylamide gels. Probably due to the higher purity, the bacterially purified MIA protein was slightly more active than protein recovered from transient eukaryotic expression and as active as the original protein purified from HTZ-19 supernatants. Both recombinant proteins were of identical size, as estimated by polyacrylamide gel electrophoresis. These data indicate that MIA function does not require further posttranslational modifications, such as glycosylation. We also found that the bacterially expressed MIA protein refolded efficiently in a phosphate buffer after proteolytic cleavage from the fusion protein. Therefore, the expression strategy we describe in this report yields physiologically active MIA protein at a purity that will be required for potential application *in vivo*.

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