Mimp, a Mitochondrial Carrier Homologue, Inhibits Met-HGF/SF-Induced Scattering and Tumorigenicity by Altering Met-HGF/SF Signaling Pathways

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Abstract

We have recently shown that Mimp, a mitochondrial carrier protein homologue, is induced by Met-hepatocyte growth factor/scatter factor (HGF/SF) signaling and decreases the mitochondrial membrane potential in DA3 mammary adenocarcinoma cells. We show here that induction of Mimp leads to growth arrest in response to HGF/SF by arresting cells at the S phase of the cell cycle. Induction of Mimp or its transient expression does not lead to apoptosis. Mimp also attenuates HGF/SF-induced cellular scattering in vitro and tumor growth in vivo. The exogenous induction of Mimp at levels similar to its endogenous induction by HGF/SF increases the level of the Met protein and its phosphorylation by HGF/SF but reduces the levels of Shc and prevents the HGF/SF-induced tyrosine phosphorylation of Grb2 and Shc. In contrast, the level of phosphatidylinositol 3-kinase (PI3K) increases following Mimp induction and the level of phosphorylated PI3K in response to HGF/SF is unaffected by the exogenous induction of Mimp. Moreover, exogenous Mimp prevents the HGF/SF-induced transcription of the serum response element-luciferase reporter gene. Our results show that Mimp expression reduces Met-HGF/SF-induced proliferation and scattering by attenuating and altering the downstream signaling of Met. These data show a new link between a tyrosine kinase growth factor receptor and a mitochondrial carrier homologue that regulates cellular growth, motility, and tumorigenicity. (Cancer Res 2006; 66(17): 8687-97)

Introduction

Met, a heterodimeric receptor tyrosine kinase, is expressed in a wide variety of normal and malignant cells. Activation of Met by its ligand hepatocyte growth factor/scatter factor (HGF/SF) can lead to many different biological outcomes, including proliferation, cell survival, scattering, invasion, and angiogenesis as well as differentiation and apoptosis (1). The Met-HGF/SF signaling pathway has been implicated in physiological processes of embryonic development and differentiation as well as in pathological cancerous transformation and metastasis (2).

Binding of HGF/SF to Met leads to autophosphorylation of several tyrosine residues in the tyrosine kinase domain of the receptor and at its docking site (3). The multivariate docking site of Met recruits adapter signaling molecules, such as Grb2, Shc, Gab1, and Crk/CRKL (4), and signaling transducers, such as phosphatidylinositol 3-kinase (PI3K), signal transducer and activator of transcription 3 (Stat3), phospholipase Cγ, and Src (5). Recruitment of Shc and Grb2 (either directly or indirectly) to the Met docking site has been implicated in Ras activation and Met-induced proliferation and migration (6). The p85 regulatory subunit of PI3K can associate directly with Met or through the adapter protein Gab1 (7), and its activation has been implicated in Met-HGF/SF-induced protection from apoptosis (8).

Recently, we reported the cloning of a novel gene, designated mimp (Met-induced mitochondrial protein), which is up-regulated in NIH 3T3 cells constitutively expressing both Met and HGF/SF (9). Mimp was shown to be induced by the signaling pathway of Met-HGF/SF in several cell lines, among them, the murine adenocarcinoma cell line DA3. Mimp is expressed in a wide range of tissues with an expression pattern similar to that of Met (9). Mimp, which is also designated mitochondrial carrier homologue 2, was identified in cDNA libraries of CD34+ hematopoietic stem/progenitor cells (10) and in expressed sequence tag libraries of mouse blastocysts (11). It was also purified from mitochondria of normal heart tissue (12), verifying its mitochondrial localization. In addition, mimp mRNA was increased 2-fold in human skeletal muscle in response to insulin (13) and was recently shown to be a target of tBid in cells signaled to die by tumor necrosis factor α (14).

Mimp encodes a 33-kDa protein that has sequence and structural homology to the family of mitochondrial carrier proteins and was shown to localize to the mitochondria (9, 14). Mimp and another novel gene product, designated presenilin-associated protein [PSAP, also known as mitochondrial carrier homologue 1 (mcht1); ref. 15], form a subfamily of mitochondrial carrier homologues, having 47% identity and 65% similarity in their amino acid sequences. Recently, PSAP was shown to be a proapoptotic mitochondrial protein and was suggested to be involved in presenilin-1-regulated apoptotic cell death cascades (16).

Our previous work showed that exogenous induction of Mimp leads to mitochondrial depolarization in Mimp-inducible DA3 (iMimp-DA3) cells (9). We now present a detailed characterization of the effect of Mimp on the cellular phenotype of DA3 cells and on their biological response to HGF/SF. Our results show that induction of Mimp attenuates the cellular response to HGF/SF in terms of growth and motility in vitro and in terms of tumorigenicity in vivo, in parallel with alterations in the Met signaling cascade.
**Materials and Methods**

**Cells.** DA3 (17), iMimp-DA3 (9), T47D, MBA-231, MBA-433, 9L (obtained from American Type Culture Collection, Bethesda, MD), and 293T cells were grown in DMEM supplemented with 10% heat-inactivated FCS (Life Technologies, Gaithersburg, MD). The generation of iMimp-DA3 cells was described elsewhere (9). Mimp was induced in these cells by incubation with different concentrations of the tetracycline derivative doxycycline (Sigma, St. Louis, MO). In general, all of the cell experiments in vitro were done in triplicate or quadruplicate and repeated at least four times.

**Plasmids, reagents, and commercial antibodies.** The cloning of Mimp and the construction of pEGFP-C1 (Clontech, Palo Alto, CA) and pcDNA3.1 and pcDNA3.1-His (Invitrogen, Carlsbad, CA) fusion vectors were described elsewhere (9). The protein products of these plasmids are designated GFP-Mimp, pcDNA-Mimp, and pcDNA-Mimp-His, respectively. Recombinant human HGF/SF was purified from the supernatant of transfected NIH 3T3 cells that overproduce this growth factor (18). HGF/SF concentrations are presented as scatter units/mL determined by the ability of the factor to scatter Madin-Darby canine kidney cells (MDCK) (units/mL). The mitochondrial membrane potential (MMP)–sensitive dye JC-1 was purchased from Molecular Probes (Eugene, OR). The trypan blue exclusion and immunoprecipitation was P20 anti-phosphotyrosine (Transduction Laboratories, Lexington, KY). The primary antibodies used for Western blot were monoclonal anti-polyhistidine (dilution 1:3,000; Sigma), monoclonal anti-Grb2 (dilution 1:500; Transduction Laboratories), mouse monoclonal anti-cdc2 (Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal anti-actin (dilution 1:500; Chemicon, Temecula, CA), polyclonal anti-p27 (dilution 1:1,000; Cell Signaling, Danvers, MA), and polyclonal antibodies anti-Met (dilution 1:200), anti-PI3K (dilution 1:200), anti-Shc (dilution 1:200), anti-p21 (dilution 1:800; all from Santa Cruz Biotechnology). The secondary antibodies were horseradish peroxidase (HRP)–conjugated anti-rabbit IgG (1:4,000) and anti-mouse IgG (1:2,000; both from Jackson ImmunoResearch Laboratories, West Grove, PA). The primary antibodies used for immunofluorescence were mouse monoclonal anti–cytokeratin 19 (dilution 1:10; Amersham, Piscataway, NJ) and 6H2.B4 mouse monoclonal anti–cytochrome c (PharMingen, San Diego, CA), and the secondary antibody was FITC-conjugated donkey anti-mouse IgG (dilution 1:150; Jackson ImmunoResearch Laboratories).

**Generation of polyclonal anti-Mimp antibodies.** The Mimp-pET28a plasmid was transformed into BL21 (DE3) plysS-competent bacteria, and Mimp-His expression was induced by isopropyl-β-thiogalactopyranoside (1 mM IPTG; 1 mmol/L) for 3 hours. The bacterial pellet was lysed under denaturing conditions (8 mol/L urea, 0.1 mol/L NaH2PO4, Tris-HCl (pH 8.0)), and the protein was extracted from the denatured supernatant on 8 mol/L urea, 0.1 mol/L NaH2PO4, Tris-HCl (pH 8.0), and the protein was extracted from the denatured supernatant on N2–NTA beads according to the protocols of Qiagen (Chatsworth, CA). Mouse lysates were analyzed by Western blot using anti-histidine antibodies for the appearance of the purified protein detected as a band at a size of ~33 kDa. The fractions containing the protein underwent dialysis against decreasing concentration of urea. After obtaining sufficient purified protein, two rabbits were immunized with an emulsion of purified protein and complete Freund’s adjuvant according to the protocols detailed in ref. 19. When the appearance of Mimp-specific antibodies was detected and no increase in antibody titer occurred between two consecutive injections, the rabbits were exsanguinated. For purification, the sera were absorbed with lyophilized BL21 bacterial powder to get rid of antibodies generated against bacterial epitopes and then further purified with ammonium sulfate (19). The titer of anti-Mimp antibodies in the sera was assessed by Western blot, and the sensitivity and specificity of the antibody were determined (results not shown).

**DNA transfection and 5,6-diamidino-2-phenylindole staining.** Cells were transiently transfected using the Lipofectamine reagent kit (Life Technologies) as suggested by manufacturer. For visualization of the nucleus, cells were fixed with cold absolute methanol for 10 minutes, permeabilized with cold acetone for 10 minutes, and stained with 4’,6-diamidino-2-phenylindole (DAPI; Sigma) for 20 minutes.

**Cell viability assay.** Cell viability was measured using the crystal violet cell staining technique (20). Briefly, cells were grown with different concentrations of doxycycline and HGF/SF for 5 days in 96-well plates. Cells were then washed with PBS, fixed with 4% paraformaldehyde, and stained with a dye containing 0.1% crystal violet in H2O for 20 minutes. Cells were then destained under running water for ~30 minutes and lysed in 0.2% Triton X-100 for 2 hours, and the absorbance was measured at 595 nm. For determination of the cellular doubling time, 106 cells were plated in identical plates and counted during the 5 following days. The slope of the graph depicting the logarithm of the number of cells in time was extrapolated by regression and the doubling time was calculated as log2/ slope.

**Cell cycle and apoptosis analysis.** For assessing cell cycle distribution, DA3 cells were synchronized at G0, following growth in starvation medium (0.1% FCS in DMEM) for 48 hours. Cells were then seeded with or without doxycycline (100 ng/mL) and allowed to grow for 5 days. HGF/SF was added to the growth medium for the last 24 hours, and the cells were fixed in 70% ethanol, washed, stained with 50 μg/mL propidium iodide (PI; Sigma), and analyzed using the fluorescence-activated cell sorting (FACS; FACSort, Becton Dickinson, Franklin Lakes, NJ). For double staining with PI and bromodeoxyuridine (BrdUrd), BrdUrd was added to the medium of growing cells 1 hour before fixation, and the preparation of cells for FACS analysis was done using a special cell proliferation kit (Amersham). FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) was used as the secondary antibody and PI was added at the final stage. For assessing apoptosis, cells were grown without synchronization with doxycycline and HGF/SF and then fixed with PI. As a positive control for apoptosis, cells were either irradiated with UV light (254 nm) at 18 J/m2 or treated with 1 μmol/L staurosporine as described previously (21).

**Serum response element-luciferase assay.** iMimp-DA3 cells were plated in 24-well plates and cotransfected with the serum response element (SRE)-luciferase (SRE-LUC) reporter plasmid and the internal control phRL-TK plasmid carrying the Renilla luciferase gene. Doxycycline was added for 3 days, and HGF/SF (80 units/mL) was added for an additional 24 hours. Cells were lysed and cell lysates were processed by using the Dual-Luciferase Assay kit (Promega, Madison, WI) according to the manufacturer’s protocol. The luciferase activity was normalized relative to Renilla for each well and the average value of six wells was compared with transfected nontreated cells.

**Scatter assay.** iMimp-DA3 and DA3 cells were grown with different concentrations of doxycycline for 2 days to allow for Mimp expression, harvested, and seeded in a scatter assay as described previously (22, 23). HGF/SF was added to quadruplicate wells in serial dilutions of two, starting from a concentration of 8,000 units/mL. Cells were allowed to grow overnight and were then fixed with methanol, air-dried, and stained with Giemsa. The concentration of HGF/SF that no longer led to cell scattering was documented by two independent observers who were unaware of the experimental setup.

**Generation and measurements of tumors in vivo.** In vivo tumorigenicity of the parental DA3 cells and iMimp-DA3 cells was assessed by orthotopic injection of 106 cells to the mammary pads of 20 female BALB/C mice (6-8 weeks old). Ten mice from each group received regular water and 10 mice received water containing 2 mg/mL doxycycline. Mice were monitored for tumor growth once weekly by caliper measurement and ultrasonographic measurement using a 15L8 linear transducer (15 MHz, Acuson, Mountain View, CA). Gray-scale sonography was done every 7 days over 21 days, and each animal was evaluated for tumor presence, size, and location. Imaging settings were standardized and unchanged throughout the experiment. No major near-field artifacts were encountered. Images were obtained by experienced sonographers who were unaware of the treatment status of the animals. The in vivo experiment was done thrice with almost identical results. All the animal work fully complied with the Van Andel Research Institute Institutional Animal Care and Use Committee requirements.

**RNA preparation, Northern blot analysis, and cDNA array analysis.** RNA was prepared using the Tri-reagent kit (Sigma) and RNA concentration was measured by absorbance at 260 nm. Northern blotting was done as described in Sambrook et al. (24). The radioactive 32P DNA probe was prepared using a purified PCR product of Mimp with the NEBlot kit.
Mimp Inhibits Met-HGF/SF-Induced Growth and Motility

Results

Characterization of Mimp induction in iMimp-DA3 cells. We have reported previously the generation of an inducible system based on DA3 mammary adenocarcinoma cells, in which Mimp is induced by the antibiotic doxycycline (9). We used the Mimp-specific antibody we have generated to show that the addition of doxycycline to the growth medium leads to induction of the Mimp protein. Actin served as an internal loading control to the experiment, and densitometric analysis and normalization of the Mimp/actin ratio relative to noninduced cells allowed quantification of the extents of induction (Fig. 1A). We then compared the levels of endogenous induction of Mimp by HGF/SF and the levels of its exogenous induction by doxycycline at both the mRNA and the protein levels. 18S rRNA and actin served as internal loading controls to the experiments, respectively (Fig. 1B and C). The level of mimp mRNA increased in response to HGF/SF, doxycycline, and their combination as shown previously (9). The increase in mimp mRNA in response to doxycycline was dose dependent (Fig. 1B). In contrast, the extent of Mimp protein induction in the presence of 100 ng/mL doxycycline was similar to its endogenous induction by HGF/SF and did not further increase in the presence of 500 ng/mL doxycycline (Fig. 1C).

Effect of Mimp induction on cellular growth. To study the effect of Mimp induction on cellular growth, iMimp-DA3 cells were plated with different concentrations of doxycycline for 5 days in the presence or absence of HGF/SF. Cell number was assessed using the crystal violet cell staining assay (Fig. 2A). Mimp induction alone did not alter cellular growth. Interestingly, whereas HGF/SF led to a small nonsignificant increase in cell number in noninduced cells, it led to a significant inhibition of growth in Mimp-induced cells. The extent of the inhibition was dependent on the dose of doxycycline (Fig. 2A).

For determining the cellular doubling time, iMimp-DA3 cells were plated with varying concentrations of doxycycline with or without HGF/SF and then counted at different time points for 1 week. The doubling time of Mimp-inducible cells increased from ~21 to 26 or 29 hours in presence of 200 or 500 ng/mL doxycycline, respectively (Fig. 2B). HGF/SF treatment did not lead to a significant change in the cellular doubling time in noninduced cells but brought about a major increase in the cellular doubling time in Mimp-induced cells at doxycycline concentrations of 500 ng/mL (59 hours; Fig. 2B). Doxycycline treatment did not affect cell number or doubling time in either the absence or the presence of HGF/SF in the parental noninducible DA3 cell line (results not shown). These results indicate that inducible Mimp itself only slightly increases the cellular doubling time but leads to a significantly prolonged doubling time in response to HGF/SF.

These results brought us to compare the levels of endogenous Mimp in several other Met-expressing cell lines that differ in their proliferative response to HGF/SF. Three human breast cancer cell lines T47D, MDA-MB-231, and MDA-MB-435 and the rat glioma cell line 9L were grown in the presence and absence of HGF/SF for 5 days, and cell number was assessed using the crystal violet cell staining method. In parallel, total cellular protein was produced from identical plates and subjected to Western blot using the anti-Mimp polyclonal antibodies. In the glioma cell line 9L, HGF/SF led to proliferation; in the three breast cancer cell lines, it led to a decrease in cell number (Fig. 2C). Interestingly, the amount of endogenous Mimp was significantly lower in 9L than in the other three cell lines. In contrast, an increase in the levels of Mimp protein in response to HGF/SF was observed only in 9L cells (Fig. 2D).

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did not alter the cell cycle distribution (Fig. 3A, a). In the iMimp-DA3 cells, HGF/SF treatment had the same effect on the cell cycle as in the control cells in the absence of doxycycline. Mimp induction at 100 ng/mL doxycycline led to a significant increase in the percentage of cells in S phase, and HGF/SF treatment of Mimp-induced cells led to a profound increase in the percentage of cells in S phase (Fig. 3A, b). Next, double staining with PI and BrdUrd was done, because PI staining allows monitoring of the cell cycle

Figure 1. Time and dose response of Mimp induction in iMimp-DA3 cells. A, a, time response of Mimp induction following 0, 8, and 24 hours of treatment with 100 ng/mL doxycycline using the anti-Mimp polyclonal antibody. Actin served as an internal loading control. b, densitometric quantification of Mimp/actin ratio normalized relative to noninduced cells. B, a, Northern blot analysis of iMimp-DA3 treated with 0, 100, and 500 ng/mL doxycycline (dox) for 2 days and then treated with HGF/SF for 24 hours using a mimp-specific probe. rRNA served as an internal loading control. b, densitometric quantification mimp/rRNA ratio normalized relative to noninduced nontreated cells. C, a, Western blot analysis using extracts generated from identical plates as in (B) using the anti-Mimp polyclonal antibody. Actin served as an internal loading control. b, densitometric quantification Mimp/actin ratio normalized relative to noninduced nontreated cells.

Figure 2. Mimp leads to growth retardation in response to HGF/SF. A, iMimp-DA3 cells were grown in the presence of varying concentrations of doxycycline with or without HGF/SF (80 units/mL) for 5 days. Cell number was determined using the crystal violet cell staining technique. The absorbance was normalized relative to control noninduced cells. B, doubling time of iMimp-DA3 cells was determined with and without HGF/SF in the presence of different concentrations of doxycycline. C, 9L, MDA-MB-231, MDA-MB-435, and T47D cells were grown in the presence and absence of HGF/SF for 5 days and the cell number was determined using the crystal violet cell staining technique. The absorbance was normalized for each cell line relative to control nontreated cells. D, total cellular protein was produced from identical plates as in (C) and subjected to Western blot using anti-Mimp antibodies. The amount of total cellular actin served as an internal loading control. *, P < 0.05; **, P < 0.01; ***, P < 0.005; ****, P < 0.001.
distribution of the cells, whereas BrdUrd staining detects the subpopulation of cells that are actively synthesizing DNA. This analysis shows that the increase in the percentage of cells at the S phase in response to HGF/SF treatment was accompanied by an increase in the percentage of BrdUrd-positive cells for each cell sample were determined. The ratio between the latter and the former was calculated. Under these conditions, the BrdUrd-positive cells accounted for 63% of all cells at the S and G2-M phases relative to only 20% under control conditions. However, the increase in S phase occurring in response to Mimp induction and to HGF/SF treatment in the presence of Mimp was not accompanied by an increase in the percentage of BrdUrd-positive cells (Fig. 3B). These results suggest that both HGF/SF and Mimp drive DA3 cells into S phase, whereas HGF/SF treatment leads to active DNA synthesis and Mimp induction leads to accumulation of cells in S phase (i.e., DNA synthesis is inhibited or altogether arrested). This effect is greatly enhanced when HGF/SF is given to Mimp-expressing cells.

Gene expression analysis using “Atlas” cDNA arrays was done to determine which cell cycle transcripts are altered during the S-phase arrest. Surprisingly, the level of cdc2 mRNA was increased in response to HGF/SF in absence of Mimp but was significantly decreased in response to HGF/SF in the presence of Mimp (Fig. 3C, a). Western blot analysis showed the same pattern of expression at the protein level (Fig. 3C, b). In contrast, the levels of the cell cycle inhibitors p21 and p27 were elevated in the presence of Mimp irrespective of HGF/SF treatment (Fig. 3D). Mimp induction does not lead to apoptosis. We have shown previously that Mimp localizes to the mitochondria and decreases the MMP in iMimp-DA3 cells (9). Because a decrease in mitochondrial potential is an early event in apoptosis (26), we assessed whether induction of Mimp leads to apoptosis. Direct observation of cells induced to express Mimp for long periods did not reveal any morphologic features characteristic of apoptosis, such as membrane blebbing, cellular rounding, or nuclear fragmentation (results not shown). We determined the percentage of cells at the sub-G0 peak as a measurement of apoptosis. HGF/SF treatment decreased the percentage of cells at the sub-G0 peak by ~3-fold in noninduced cells. Induction of Mimp had no significant effect on the percentage of cells at the sub-G0 peak both without and with HGF/SF (Fig. 4).
To study the effect of Mimp induction on the cellular susceptibility to apoptosis, noninduced and Mimp-induced cells were exposed to UV light at a dosage known to induce apoptotic cell death (8) 24 hours before cell fixation. UV irradiation led to an accumulation of cells at the sub-G0 peak in both noninduced and Mimp-induced cells in a similar extent (Fig. 4B and C). Figure 4B shows representative histograms of the cells in the four different conditions, and Fig. 4C depicts the average percentage of cells at the sub-G0 peak in the absence and presence of doxycycline in nonirradiated and in UV-irradiated cells. UV irradiation for a longer period increased the percentage of sub-G0 cells in both noninduced and Mimp-induced cells without a significant difference between these two cell populations (results not shown). In concordance with this observation, a cytoplasmic DNA ladder, characteristic of cells undergoing apoptosis, was not apparent in the noninduced or Mimp-induced cells but was observed in the UV-irradiated cells under both conditions (results not shown). Last, the effect of Mimp induction on the subcellular localization of cytochrome c was studied using immunofluorescence. In both noninduced and Mimp-induced cells, cytochrome c was localized to the mitochondria without being released to the cytoplasm (Fig. 4D). This result further corroborates our observation that Mimp induction does not lead to apoptosis.

It was recently published that PSAP, which carries 47% identity and 65% similarity to Mimp, localizes to the mitochondria and leads to apoptosis following transient transfection into 293T (16). We therefore checked whether Mimp leads to apoptosis in a transient transfection assay. pEGFP-Mimp-transfected cells, which showed mitochondrial staining as observed previously (9), had normal cellular morphology with a flattened appearance.

In contrast, cells in which apoptosis was chemically induced by staurosporine showed a rounded morphology typical of cells undergoing programmed cell death. In addition, nuclear staining of pEGFP-Mimp-transfected cells with DAPI following fixation showed that the nuclei were large, round, and intact, whereas in the staurosporine-treated cells the nuclei looked condensed (Fig. 5A).

293T cells were transiently transfected with an empty pcDNA vector, pcDNA-Mimp, or pcDNA-Mimp-His plasmid. The transfection was verified by Western blot analysis with anti-His antibodies (Fig. 5B). Transient transfection with Mimp or Mimp-His did not increase the percentage of cells at the sub-G0 peak of the cell cycle relative to cells transfected with the empty pcDNA vector or to control nontransfected cells (Fig. 5B, b). Interestingly, transient transfection of 293T with pcDNA-Mimp or pcDNA-Mimp-His led to mitochondrial depolarization as shown previously for DA3 cells (9). The average red/green fluorescence JC-1 ratio of Mimp-transfected or His-Mimp-transfected cells was lower than the average ratio in control cells or cells transfected with the empty vector (Fig. 5C). These results show that although Mimp leads to a decrease in the MMP when transiently expressed in 293T it does not lead to cellular apoptosis.

Mimp induction attenuates HGF/SF-induced scattering. One of the well-documented responses of several cell lines, including DA3, to HGF/SF is cellular scattering (27). To study the effect of Mimp induction on scattering, iMimp-DA3 cells were plated in the presence of 100 or 500 ng/mL doxycycline for 2 days to allow for Mimp expression and then harvested and plated in quadruplicate

![Figure 4](https://cancerres.aacrjournals.org/content/66/17/8692/F4.large.jpg)
in the presence of serial dilutions of HGF/SF (see Materials and Methods for details). In control cells, the scattering effect of HGF/SF disappeared at a concentration of 1 unit/mL, whereas in the presence of Mimp the scattering effect of HGF/SF was no longer apparent at concentrations of 4 units/mL. Representative micrographs of cells grown in the presence of 2 units/mL HGF/SF are seen in Fig. 6A, which show that in presence of 500 ng/mL doxycycline Mimp-inducible cells grew in patches, whereas in the absence of doxycycline these cells were dispersed separately in the plate and showed a distinct spindle morphology. This could not be attributed to an effect of the antibiotic itself, as doxycycline treatment of the parental DA3 cells did not prevent their scattering in response to HGF/SF (Fig. 6A).

For further analyzing cellular morphology, cells were induced to express Mimp for 2 days and then harvested and seeded again in the presence of HGF/SF for 24 hours. Cells were fixed and part of the cytoskeletal network visualized by staining with an antibody against cytokeratin-19, an intermediate filament known to be expressed extensively in the cytoplasm of epithelial cells (28). As reported previously for MDCK (29), HGF/SF treatment of noninduced cells altered cellular morphology and cellular distribution of cytokeratin-19 (Fig. 6B). Control nontreated cells exhibited a rounded morphology, in which cytokeratin-19 formed a homogeneous cytoplasmatic mesh. HGF/SF treatment led to cellular polarization, with cells having a typical spindle shape and two long cytoplasmatic extensions. Cytokeratin-19 was mainly dispersed around the nuclei and in those extensions (Fig. 6B). Induction of Mimp altered the HGF/SF-induced polarization and redistribution of cytokeratin-19. At doxycycline concentrations of 500 ng/mL, cells treated with HGF/SF had more rounded and less “spindle-like” morphology, and the cytokeratin-19 was distributed throughout the cytoplasm in a “mesh-like” pattern (Fig. 6C). These results indicate that Mimp attenuates HGF/SF-induced changes in cellular morphology, which are an essential step in the process of cellular migration and scattering.

**Induction of Mimp decreases tumor growth in vivo.** iMimp-DA3 and DA3 cells were injected into the mammary pads of female BALB/c mice. The mice were then divided arbitrarily to two.
subgroups, one receiving regular drinking water and the other receiving drinking water containing 2 mg/mL doxycycline. The development of mammary tumors was monitored once weekly by caliper measurement and ultrasonographic measurement. Addition of doxycycline to the drinking water did not affect tumor growth rate in the noninducible DA3 cells as assessed by ultrasonographic measurement (Fig. 7A, left). In contrast, in the Mimp-inducible cells, the tumors in the group receiving doxycycline grew significantly slower than in the control group (Fig. 7A, right). For each time point, the average tumor size of the group receiving doxycycline was 30% ($P = 0.002$) to 60% ($P = 0.006$) of the control group. In addition, the tumors from the control group ruptured on average earlier than tumors of the mice receiving doxycycline, indicating that necrosis of the tumor mass occurred earlier in the control group than in the group receiving doxycycline. The complementary approach of caliper measurements of tumor size yielded similar results (results not shown). Representative micrographs from the ultrasonographic imaging of tumors from iMimp-DA3 cells are seen (Fig. 7B), which show that the tumor of the mouse receiving regular drinking water was significantly larger at each time point than the tumor of the mouse receiving doxycycline.

**Induction of Mimp alters Met-HGF/SF-induced signaling.**

The differential response to HGF/SF in the presence of Mimp motivated us to check the expression and phosphorylation levels of several known signaling molecules downstream of Met. iMimp-DA3 cells were treated with different concentrations of doxycycline for 2 days, serum starved for 24 hours, and then treated with HGF/SF for 20 minutes. The protein levels and the extent of their phosphorylation were assessed by Western blot analysis or immunoprecipitation followed by Western blot, respectively (Fig. 8A). The results were quantified by densitometry and normalized relative to noninduced nontreated cells (Fig. 8B).

We observed a dose-dependent increase of Met protein levels as a result of Mimp induction (Fig. 8, top). In addition, the levels of phosphorylated Met in response to HGF/SF were higher in Mimp-induced cells relative to noninduced cells, although the basal levels of phosphorylated Met were similar in all three conditions (Fig. 8A, top). The attenuation in the HGF/SF-induced cellular response could not therefore be attributed to a decrease in the initial phosphorylation of the Met receptor.

Grb2 was phosphorylated in response to HGF/SF treatment. Induction of Mimp decreased the basal level of phosphorylated Grb2 in a dose-dependent manner. It also prevented the
HGF/SF-induced elevation of phosphorylated Grb2 (Fig. 8B, middle). The levels of the Shc adapter protein were decreased in the presence of both concentrations of doxycycline, and its phosphorylated form was decreased following Mimp induction at 500 ng/mL doxycycline (Fig. 8B, middle). In the parental DA3 cells, there was no change in the levels of Shc or phosphorylated Shc in the presence of 100 and 500 ng/mL doxycycline (results not shown).

In contrast, an increase in the levels of PI3K was observed following Mimp induction. The basal level of PI3K phosphorylation was reduced in Mimp-induced cells, but it returned to control levels following HGF/SF treatment at both doxycycline concentrations (Fig. 8B, bottom). All these results were obtained with the same two membranes, thus ruling out the possibility that these differences stem from differences in the extent of protein loading.

To study whether the decrease in the levels of signaling molecules in presence of Mimp is due to transcriptional regulation, iMimp-DA3 cells were transiently transfected with a vector containing the reporter gene luciferase under a promoter containing SRE. As shown previously, HGF/SF induced expression from the SRE-LUC vector (30). Induction of Mimp prevented the HGF/SF-induced SRE-LUC expression (Fig. 8C).

**Discussion**

In this work, we use an inducible system of Mimp expression in DA3 adenocarcinoma cells. In this model system, there is a tight regulation on the induction of the Mimp protein in response to doxycycline and HGF/SF that is not seen at the transcriptional level. As a consequence, the levels of the inducible protein in our system are very similar to its endogenous levels in response to induction by HGF/SF. The mechanism of such post-transcriptional regulation is currently not known. Interestingly, a discrepancy between mRNA and protein expression levels was also described for UCP2 and UCP3, two other proteins from the mitochondrial carrier family (31, 32), and the existence of post-transcriptional mechanisms that regulate the expression of UCP2 and UCP3 was proposed (31, 32). The tight post-transcriptional regulation on the expression of Mimp, UCP2, UCP3, and possibly other carriers may indicate the importance of maintaining an adequate amount of these proteins in the cell.

The work presented here characterizes the effects of Mimp induction on the biological responses of DA3 cells to HGF/SF. Induction of Mimp does not decrease cell proliferation rate and only slightly elongates the cellular doubling time but does lead to a
G2-M phases of the cell cycle in anaplastic thyroid cancer cells in percentage of cells in the S phase (35). Last, the cancer drug irradiation and does not lead to cytochrome does not render cells more susceptible to apoptosis by UV. In contrast to mtch1, Mimp does not lead to apoptosis. The carrier homologue most similar to Mimp, is a proapoptotic protein (16). The mitochondrial carrier protein homologue that is not in itself toxic to cells inhibits tumor growth. mtch1, the mitochondrial protein (33, 34). In both cases, the decrease in cdc2 was accompanied by an increase in the levels of the p21 inhibitor (33, 34). In addition, a significant decrease in total cdc2 protein was observed in prostate cancer cells following incubation with IGFBP3, which also leads to a significant reduction in [3H]thymidine incorporation and to an increase in the percentage of cells in the S phase (35, Last, the cancer drug STI571 was shown to lead to accumulation of cells at the S and G2-M phases of the cell cycle in anaplastic thyroid cancer cells in a mechanism that involved both a decrease in cdc2 protein and an increase in p21 and p27 (36). The alterations in the cell cycle proteins and the S-phase arrest in response to Mimp can explain the observed elongation of the doubling time and the decrease in cell growth in response to HGF/SF. To our knowledge, our results are the first to show S-phase arrest following induction of a mitochondrial protein.

The observation that exogenous induction of Mimp attenuates the proliferative response to HGF/SF in DA3 brought us to check its endogenous levels in cell systems that differ in their proliferative response to HGF/SF. Interestingly enough, the cell line that responded to HGF/SF in proliferation had significantly lower levels of Mimp than the three cell lines that responded to HGF/SF in growth arrest. This correlative observation may suggest that the basal levels of Mimp might be one of the variables that affect the nature of the cellular response to HGF/SF either directly or indirectly.

Currently, little is known about the involvement of proteins from the mitochondrial carrier superfamily in cellular transformation. This work shows for the first time that induction of a mitochondrial carrier protein homologue that is not in itself toxic to cells inhibits tumor growth. mtch1, the mitochondrial carrier homologue most similar to Mimp, is a proapoptotic protein (16). In contrast to mtch1, Mimp does not lead to apoptosis. Moreover, HGF/SF has an antiapoptotic effect in DA3 cells as has also been described for other cells (8, 37, 38), and this effect is preserved in the presence of Mimp. In addition, Mimp expression does not render cells more susceptible to apoptosis by UV irradiation and does not lead to cytochrome c release from the mitochondria. Overexpression of Mimp in 293T cells does not lead to apoptosis, although it does lead to depolarization of the mitochondria.

Mimp also inhibits HGF/SF-induced scattering, because in its presence the concentrations of HGF/SF needed for scattering are higher than in its absence. The process of cell scattering includes the phases of cell spreading, cell-cell dissociation, and cell migration. Disassembly of cell-cell junctions occurs during certain physiologic conditions throughout development as well as under pathologic circumstances, such as tumor invasion and metastasis (1). Thus far, a mitochondrial protein has not been implicated in inhibition of cellular scattering.

Induction of Mimp at levels similar to its physiologic induction by HGF/SF leads to profound changes in the total levels of several signaling molecules downstream of Met and in their phosphorylation patterns. The levels of Shc are decreased when Mimp is induced, and the extent of its tyrosine phosphorylation is greatly reduced following induction of Mimp with or without HGF/SF treatment. Shc is a substrate of Met and works as an amplifier of the motogenic as well as of the mitogenic response to HGF/SF (6). Shc also recruits Grb2 to Met (6). Grb2, an important adaptor protein for Met-HGF/SF, was found to mediate many of the HGF/SF-induced responses (5, 39), especially proliferation (40), by binding either directly to the docking site of Met or through Shc (6). Grb2 is phosphorylated in response to HGF/SF in DA3 cells. The induction of Mimp reduces the extent of Grb2 phosphorylation in a dose-dependent manner. It was shown that antagonists to Grb2-SH2 domain interactions lead to potent blockade of HGF/SF-stimulated cell motility, matrix invasion, and branching morphogenesis (41). The induction of Mimp leads to a similar alteration in Grb2 signaling and to a similar attenuated response to HGF/SF. In contrast, Mimp induction increases both the levels of Met and the extent of its phosphorylation in response to HGF/SF. These results indicate that the Mimp-induced effects on the signaling pathway occur downstream of the receptor.

The levels of the protein PI3K are elevated when Mimp is induced. Following Mimp induction, PI3K phosphorylation is decreased but increases to control levels following HGF/SF treatment. It therefore seems that the cell “maintains” steady-state levels of phosphorylated PI3K in presence of both low and high levels of Mimp by elevating the basal protein levels. PI3K has been implicated in the antia apoptotic response to HGF/SF in several cell types (8). We propose that the maintenance of a constant level of phosphorylated PI3K in the presence of HGF/SF and Mimp is part of what allows the preservation of the antia apoptotic effect of HGF/SF in the presence of Mimp.

Activation of Met by HGF/SF leads to activation of the downstream signaling pathway of Ras-Raf-1-Erk1/Erk2-Elk-1, the last being a transcription factor that binds to SRE (30). This signaling pathway is well known to be involved in cellular proliferation, and it has been shown in the past that HGF/SF induces the expression of SRE-LUC in HeLa cells (30). We show a similar induction of SRE-LUC in response to HGF/SF in several cell types (8). We propose that the maintenance of a constant level of phosphorylated PI3K in the presence of HGF/SF and Mimp is part of what allows the preservation of the antia apoptotic effect of HGF/SF in the presence of Mimp.

In summary, our current work suggests that induction of exogenous Mimp at the mitochondria leads to alterations in the transcription from SRE-containing promoters, thus changing the expression pattern of signaling molecules. These effects of Mimp lead to the observed decrease in growth and in motility in response to HGF/SF without leading to apoptosis. We hypothesize that the endogenous induction of Mimp by Met-HGF/SF serves as a negative regulatory mechanism that restrains their signaling.
thus lowering the potential harmful outcome of activation of this receptor.

Met is overexpressed in many human tumors (1, 42) and is a potential target for antitumor therapy (1). Identifying proteins that serve as natural negative regulators of this important signaling pathway may shed light on the molecular mechanisms of Met-induced tumorigenicity and metastasis and may aid in finding efficient ways to block this activity.

References


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Mimp, a Mitochondrial Carrier Homologue, Inhibits Met-HGF/SF-Induced Scattering and Tumorigenicity by Altering Met-HGF/SF Signaling Pathways

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