Mitogen-inducible gene 6 is an endogenous inhibitor of HGF/Met-induced cell migration and neurite growth

Guido Pante,1 Jane Thompson,2 Fabienne Lamballe,3 Tomoko Iwata,4 Ingvar Ferby,1 Francis A. Barr,5 Alun M. Davies,6 Flavio Maina,3 and Rüdiger Klein1

1Department of Molecular Neurobiology, Max Planck Institute of Neurobiology, 82152 Munich-Martinsried, Germany
2Fujisawa Institute of Neuroscience, Edinburgh EH8 9JE, Scotland, UK
3INSERM UMR 623, Developmental Biology Institute of Marseille, 13288 Marseille, Cedex 09, France
4Division of Cancer Sciences and Molecular Pathology, Faculty of Medicine, University of Glasgow, Beatson Laboratories, Bearsden, Glasgow G61 1BD, Scotland, UK
5Intracellular Protein Transport Independent Junior Research Group, Max Planck Institute of Biochemistry, 82152 Munich-Martinsried, Germany
6School of Biosciences, Cardiff CF10 3US, Wales, UK

Introduction

The signals that regulate the development of organs need to act for a limited duration in the right place and time. To avoid signaling errors that lead to aberrant cellular behavior and disease, cellular mechanisms have evolved to ensure that appropriate parameters of a signal are received and maintained for the correct time. Hepatocyte growth factor (HGF)/scatter factor controls cell migration, growth, and differentiation in several embryonic organs and is implicated in human cancer. The physiologic mechanisms that attenuate Met signaling are not well understood. Here we report a mechanism by which mitogen-inducible gene 6 (Mig6; also called Gene 33 and receptor-associated late transducer) negatively regulates HGF/Met-induced cell migration. The effect is observed by Mig6 overexpression and is reversed by Mig6 small interfering RNA knock-down experiments; this indicates that endogenous Mig6 is part of a mechanism that inhibits Met signaling. Mig6 functions in cells of hepatic origin and in neurons, which suggests a role for Mig6 in different cell lineages. Mechanistically, Mig6 requires an intact Cdc42/Rac interactive binding site to exert its inhibitory action, which suggests that Mig6 acts, at least in part, distally from Met, possibly by inhibiting Rho-like GTPases. Because Mig6 also is induced by HGF stimulation, our results suggest that Mig6 is part of a negative feedback loop that attenuates Met functions in different contexts and cell types.

Hepatocyte growth factor (HGF)/Met signaling controls cell migration, growth and differentiation in several embryonic organs and is implicated in human cancer. The physiologic mechanisms that attenuate Met signaling are not well understood. Here we report a mechanism by which mitogen-inducible gene 6 (Mig6; also called Gene 33 and receptor-associated late transducer) negatively regulates HGF/Met-induced cell migration. The effect is observed by Mig6 overexpression and is reversed by Mig6 small interfering RNA knock-down experiments; this indicates that endogenous Mig6 is part of a mechanism that inhibits Met signaling. Mig6 functions in cells of hepatic origin and in neurons, which suggests a role for Mig6 in different cell lineages. Mechanistically, Mig6 requires an intact Cdc42/Rac interactive binding site to exert its inhibitory action, which suggests that Mig6 acts, at least in part, distally from Met, possibly by inhibiting Rho-like GTPases. Because Mig6 also is induced by HGF stimulation, our results suggest that Mig6 is part of a negative feedback loop that attenuates Met functions in different contexts and cell types.

Correspondence to Rüdiger Klein: rklein@neuro.mpg.de

Abbreviations used in this paper: Ack, activated Cdc42-associated tyrosine kinase; Cdc42*, dominant-active Cdc42; CRIB, Cdc42/Rac interactive binding; FGF2, fibroblast growth factor 2; Grb2, growth factor receptor–bound protein 2; HGF, hepatocyte growth factor; LacZV5, V5 epitope–tagged /H9252-galactosidase; Mig6, mitogen-inducible gene 6; Mig6FL, full-length Mig6; Mig6FL–V5, SDF-1, stromal cell–derived factor 1; V5 epitope–tagged versions of Mig6; PI3, phosphatidylinositol 3; siRNA, small interfering RNA.

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such as mitogen-inducible gene 6 (Mig6; see below next paragraph) (for review see Pirone et al., 2001).

Signaling by receptor tyrosine kinases requires a counterbalance by negative signaling events to ensure that appropriate thresholds of receptor signals are achieved and maintained for the right length of time. Irreversible inhibition is mediated most commonly by activation-dependent protein degradation through the ubiquitin–proteasome pathway. Reversible inhibition can be achieved by protein tyrosine phosphatases, by dual-specificity MAPK phosphatases, and by phosphatase and tensin homologue proteins, which down-regulate the PI3 kinase pathway (Dikic and Giordano, 2003).

In a screen for HGF-induced changes in the transcriptome of cultured cells, we identified the Mig6 adaptor protein in a set of highly induced transcripts. Mig6 (also known as Gene 33 and receptor-associated late transducer) is considered an immediate early response gene that can be induced by a variety of external stimuli, including growth factors, cytokines, and stress factors (Wick et al., 1995; Makkinje et al., 2000; and references within). Overexpression and knock-down studies suggested that Mig6 was a selective inhibitor of EGF receptor family (also known as ErbB receptors)–mediated mitogenesis and transformation (Fiorentino et al., 2000; Hackel et al., 2001; Fiorini et al., 2002; Anastasi et al., 2005; Xu et al., 2005). Its mechanism of action, receptor specificity, and influence on other cellular activities are poorly understood or unknown.

Here we show that Mig6 is a negative regulator of HGF/Met-induced cell migration. The effect was observed by Mig6 overexpression and reversed by Mig6 small interfering RNA (siRNA) knock-down experiments, which indicates that endogenous Mig6 is part of a mechanism that inhibits Met signaling. The effect is observed in cells of hepatic origin as well as in primary neurons; this suggests that Mig6 functions across different cell lineages. Met lacks the sequence identified in EGF receptor as the Mig6 binding region (Hackel et al., 2001) and fails to bind Mig6 directly. Instead, Mig6 requires an intact CRIB domain to exert its inhibitory action, and suggests that Mig6 acts, at least in part, distal from the receptor, possibly by interacting with Rho family GTPases. Because Mig6 also is induced by HGF stimulation, our results suggest that Mig6 is part of a negative feedback loop that attenuates Met signaling in a variety of cellular functions.

Figure 1. Induction of Mig6 by HGF/Met signaling in cultured cells and coexpression of Mig6 with Met in vivo. (A–C) Northern blot analysis showing mig6 mRNA up-regulation upon HGF, FGF, or PDGF stimulation for 4 h (A and B) or the indicated times (C) in MLP29 and C2C12 cells. 18S or glyceraldehyde 3-phosphate dehydrogenase (G3PDH) mRNA levels were used as internal control. Western blot (W.B.) analysis showing Mig6 protein induction upon HGF and EGF stimulation in MLP29 cells (D) and primary hepatocytes (E). Cells were grown in 10% FBS, then stimulated with empty media, 40 ng/ml HGF, or 10 ng/ml EGF for the indicated times, lysed, and analyzed by SDS-PAGE and immunoblotting using a specific anti-Mig6 antiserum. Immunoblots for α-tubulin and Met were used as internal controls. Immunocytochemistry analysis showing Mig6 protein induction upon HGF stimulation in MLP29 cells. Cells stimulated as above with empty media (F) or with HGF (G), fixed, and stained using a specific anti-Mig6 antiserum. NT, not treated. Bar, 50 μm. (H–N) In situ hybridization analyses for mig6 and met mRNA transcripts in selected organs of E13.5 wild-type (+/+) and Met signaling-deficient (met−/−) embryos. Co-expression of mig6 and met is observed in alveoli of the lungs (Lu), liver (Li), intercostal muscle (i.m.), and body wall muscle (b.w.) (I, J, M, and N). Mig6 transcript levels are reduced in met−/− embryo lungs and liver (K). Mig6 sense probe was used as negative control on adjacent sections (H, L). Ri, ribs. Bar, 300 μm.
Results

Mig6 induction by HGF/Met signaling

Part of our efforts to understand the molecular events that control HGF/Met signaling has been to examine the expression of genes that are regulated by Met signaling. RNA from HGF-stimulated and unstimulated MLP29 cells, a liver-derived cell line that expresses physiologic levels of Met (Medico et al., 1996), was subjected to microarray analysis (Tanaka et al., 2000). Genes that showed >1.8-fold changes and whose sum of median value was >5,000 were included in the final list. Expression levels of ~100 genes were up-regulated, whereas ~30 genes were down-regulated by HGF stimulation in MLP29 cells (Tables S1 and SII; available at http://www.jcb.org/cgi/content/full/jcb.200502013/DC1). Analysis, by Northern blotting, of selected candidates on two independent cell lineages (MLP29 hepatic cells and C2C12 myoblast cells) confirmed Met-mediated regulation of most hits from the microarray analysis (Fig. S1 A).

Mig6 was chosen for further analysis because its expression was induced more strongly by HGF—4.5- to 80-fold by 4 h of HGF stimulation—than by fibroblast growth factor 2 (FGF2) or PDGF (Fig. 1, A and B). Other transcripts did not show this preference for HGF (Fig. S1 B). The reduced response to FGF2 and PDGF was not due to lack of specific receptors or downstream transducers, because both growth factors induced robust phosphorylation of ERK/MAPKs in MLP29 cells (Fig. S2; available at http://www.jcb.org/cgi/content/full/jcb.200502013/DC1). Time courses of HGF stimulation revealed that Mig6 mRNA and protein were induced half maximally after 1 h and maintained for several hours (Fig. 1, C and D). The induction of Mig6 protein by EGF was more transient than that induced by HGF (Fig. 1 D). Induction of Mig6 protein by HGF in primary hepatocytes followed delayed kinetics (Fig. 1 E). We also confirmed the induction of endogenous Mig6 protein by immunostaining of MLP29 cells (Fig. 1, F and G). We next investigated the expression of Mig6 and Met in embryonic tissues. By in situ hybridization analysis, we found that both transcripts coexpressed in alveoli of...
embryonic (E13.5) lung, in liver parenchyma, and in intercostal and body wall muscles of wild-type embryos (Fig. 1, I, J, M, and N). Consistent with Met regulating mig6 transcript levels under physiologic conditions, we found reduced levels of mig6 mRNA in embryos that expressed a signaling-deficient Met receptor (met<sup>kn</sup>) (Maina et al., 1996, 2001) (Fig. 1 K). Expression of Mig6 protein was confirmed in structures positive for mig6 mRNA, including intercostal and body wall muscles (Fig. S2).

Overexpression of Mig6 inhibits HGF-induced migration of MLP29 cells

The only cellular context in which Mig6 has been implicated is cell division. However, because HGF/Met signaling is critical for cell migration, we tested the effects of Mig6 overexpression on HGF/Met-mediated migration in the Boyden chamber migration assay. This assay tests the capacity of the cells to migrate through a porous membrane that separates upper and lower compartments. MLP29 cells were transfected with a plasmid that encodes YFP, or cotransfected with plasmids that encode full-length Mig6 (Mig6<sup>FL</sup>) and YFP (Fig. 2, A–C). We confirmed the expression of exogenous Mig6 by immunostaining of cells and immunoblotting of lysates from cells expressing V5 epitope–tagged versions of Mig6 (Mig6<sup>FL-V5</sup>) in comparison with V5 epitope–tagged β-galactosidase (LacZ<sup>V5</sup>) (Fig. 2, D–J). For each experimental condition, equal numbers of cells were seeded onto coverslips for immunocytochemical analysis, or onto the upper compartment of the Boyden chamber, and were exposed to varying concentrations of HGF or 10% FBS in the lower compartment. Cell migration through the membrane into the lower compartment was stimulated by HGF in a dose-dependent manner (unpublished data). At 40 ng/ml HGF, migration of transfected, YFP-positive cells was enhanced 30- to 40-fold over unstimulated cells (Fig. 2, O and P). Representative images of Hoechst dye–labeled cells, which migrated into the lower compartment was stimulated by HGF in a dose-dependent manner (unpublished data). At 40 ng/ml HGF, migration of transfected, YFP-positive cells was enhanced 30- to 40-fold over unstimulated cells (Fig. 2, O and P). Representative images of Hoechst dye–labeled cells, which migrated into the lower compartment of the Boyden chamber, are shown in Fig. 2 (K–N).

The presence of Mig6<sup>FL</sup> reduced HGF-stimulated cell migration by approximately threefold (Fig. 2 O, P < 0.001, t test). In contrast, cell migration that was stimulated with 10% FBS was unaffected by Mig6 overexpression (P = 0.121, t test).

Because Mig6 overexpression also reduced cell proliferation that was induced by HGF (unpublished data), we next asked whether the effect on cell migration could be secondary to reduced cell proliferation. Transfected cells were exposed to 1.6 μg/ml of the DNA polymerase inhibitor, aphidicolin, 24 h before plating onto the membrane; the assay was conducted in the presence of 1.6 μg/ml aphidicolin. Typically, aphidicolin treatment reduced the incorporation of BrdU into MLP29 cells 10-fold (unpublished data). Under these conditions, HGF-stimulated cell migration still was reduced significantly by Mig6 overexpression (P = 0.02; 10 ng/ml HGF, P < 0.0002; 40 ng/ml HGF, P < 0.01, t test).

Mig6 is a physiologic suppressor of Met-mediated cell migration

We next investigated, using RNA interference, whether endogenous Mig6 suppressed HGF/Met-mediated cell migration by increasing of cell migration of mig6 knock-down cells after HGF stimulation expressed as fold of induction over unstimulated cells. Increasing concentrations of HGF were added to the media of the lower compartment of the chamber and the cells were allowed to migrate for 16 h. Note the increase of cell migration of mig6 knock-down cells after HGF stimulation (compare panels f and g). Bar, 100 μm. (H) Quantification of migration expressed as fold of induction over unstimulated cells. Increasing concentrations of HGF were added to the lower chamber after transfection of control GFP siRNAs (black bars) or mig6 siRNAs (gray bars). 5 ng/ml HGF, P = 0.02; 10 ng/ml HGF, P < 0.0002; 40 ng/ml HGF, P < 0.01, t test.
knocking down Mig6 protein levels (Elbashir et al., 2001). MLP29 cells were transfected with siRNAs specific for GFP or mig6, and the levels of Mig6 protein were analyzed by immunostaining and immunoblotting. Mig6 siRNA, but not control GFP siRNA, specifically knocked down endogenous Mig6 immunoreactivity 96 h after transfection (Fig. 3, A and B). Mig6 siRNA also suppressed HGF-stimulated induction of Mig6 (Fig. 3 C; compare 4-h time point in the presence and absence of mig6 siRNA). The reduction of protein levels was specific for mig6, because endogenous α-tubulin and Met levels were unaffected (Fig. 3 C and not depicted). To investigate the role of Mig6 protein in cell migration, cells were transfected with mig6 siRNA oligonucleotides, and subjected to the Boyden chamber assay with different concentrations of HGF (Fig. 3). Representative images of Hoechst dye–labeled cells that migrated into the lower compartment are shown in Fig. 3 (D–G). The induction of cell migration by HGF under these conditions was less strong, yet was still dose dependent (Fig. 3 H). Quantification of migrating cells revealed that under conditions of optimal HGF concentrations, knock down of Mig6 enhanced cell migration by approximately twofold (Fig. 3 H). Similar results were obtained with a separate set of siRNA oligonucleotides (unpublished data). These findings demonstrated that Mig6 is a physiologic inhibitor of HGF/Met-mediated cell migration of MLP29 cells.

Knock-down of Mig6 also enhanced MLP29 cell proliferation (unpublished data), as previously shown in EGF-stimulated fibroblasts (Xu et al., 2005). To separate Mig6’s effects on cell proliferation from cell migration, we assayed cell migration in the presence of the cell cycle inhibitor, aphidicolin. In the presence of DMSO-containing control media, knock-down of Mig6 protein levels led to a significant increase in HGF-induced cell migration (Fig. 4, A, B, and E). This effect was not diminished by the presence of aphidicolin, if anything, it was enhanced slightly (Fig. 4, C–E). These data indicate that endogenous Mig6 blocks cell migration independently of its antiproliferative effects.

### Cortical neuron migration induced by HGF is sensitive to overexpression of Mig6

We next asked whether the functions of Mig6 were specific to cells of hepatic origin, or whether Mig6 had a more general role in controlling cell migration across different cell lineages, including neurons from the neocortex (Powell et al., 2001). To test the consequences of Mig6 overexpression, embryonic cortical neurons were transfected with a Mig6FL-V5 construct, and Mig6 expression was monitored by immunofluorescence microscopy (Fig. 5, D–G). Our modified electroporation protocol (see Materials and methods) led to transfection efficiencies of 40–70%, as judged by GFP fluorescence (Fig. 5 A). Mig6 overexpression did not affect survival (unpublished data) or differentiation of cortical neurons as judged by the expression of microtubule-associate protein 2 (Fig. 5 B,C). HGF-induced cell migration was assayed 36 h after transfection of expression plasmids that encoded GFP or Mig6FL-V5. Quantification of all (transfected and untransfected) cells on the lower face of the porous membrane revealed a three- to fourfold increase of migrated cells in the presence of HGF, and a seven- to eightfold stimulation among the GFP transfectants (Fig. 5 H). In contrast, overexpression of Mig6 completely prevented HGF from inducing cell migration (Fig. 5 H). In a separate set of experiments, we compared cells that were transfected with plasmids that encoded Mig6FL-V5 or LacZV5 in the absence or presence of HGF. 24 h after transfection, LacZV5 control cells responded to HGF with a six- to sevenfold higher migration rate (Fig. 5 I). In contrast, Mig6FL-V5-expressing cells failed to migrate (Fig. 5 I; P < 0.0001; t test). The effect of Mig6 on HGF-induced cell migration was specific, because Mig6 was unable to block cell migration in response to the chemokine stromal cell–derived factor-1 (SDF-1) (Fig. 5 I; P > 0.28, t test).

### Mig6 suppresses HGF/Met-mediated neurite growth

Besides regulating cell migration, HGF is a chemoattractant and neurite growth-promoting factor for subsets of neurons, including sympathetic neurons (Maina and Klein, 1999; Thompson et al., 2004). We next tested the effects of Mig6 on neurite...
growth of paravertebral sympathetic neurons. Most postnatal day 40 sympathetic neurons of the superior cervical ganglion survive in culture without addition of neurotrophic factors (unpublished data). Exogenous HGF induced Mig6 expression (Fig. 6 A), and stimulated outgrowth and branching of neurites. To quantify this effect, we transfected the cells with an expression plasmid that encoded YFP by way of gene gun, and used Sholl analysis (see Materials and methods) to determine neurite complexity. HGF (10 ng/ml) significantly increased neurite complexity and branching as compared with nontreated control cultures (Fig. 6, D and F). The effect was most pronounced close to the soma, whereas there was no significant increase in complexity in the longest neurites (Fig. 6 D). To investigate neurite complexity, we compared the neurite arbors of cells that were transfected with expression vectors encoding Mig6 plus YFP with cells expressing YFP alone (Fig. 6, B and C). Overexpression of Mig6 greatly reduced neurite outgrowth and branching of HGF-stimulated cells (Fig. 6 E). The effect was most pronounced close to the cell soma, where HGF had its strongest effects in comparison with control cDNAs, such as YFP (Fig. 6 E). This effect was specific for HGF-treated neurons, because in untreated neurons, expression of Mig6 did not cause a reduction of branch points (Fig. 6 F). Exogenous Mig6 also did not affect survival of HGF-treated neurons, which suggests that Mig6 specifically inhibited Met signaling toward neurite growth (Fig. 6 G). We next performed Mig6 knockdown experiments and found a modest increase in neurite length in Mig6 siRNA-treated, as compared with GFP siRNA-treated, cells. The increase in Mig6 knock-down cells was significant in neurites that extended furthest from the soma. Because the induction of endogenous Mig6 protein is delayed (see Fig. 6 A), the effect of knocking down mig6 mRNA may be
visible only in the longest neurons that took the most time to grow. These results suggest that Mig6 plays a role in suppressing neurite complexity that is induced by HGF/Met signaling. The CRIB domain of Mig6 is required for and sufficient to inhibit HGF-mediated cell migration

Mig6 was proposed to inhibit EGFR signaling by direct binding to EGFR and ErbB2—by suppressing the EGFR kinase activity—and by a receptor distal mechanism (Anastasi et al., 2003). To begin dissecting the mechanism of Mig6-mediated inhibition of cell migration, we asked whether Mig6 would directly bind Met in MLP29 cells that were stimulated with HGF. In pull-down experiments that used bacterially expressed, GST-tagged, purified, full-length Mig6, we were unable to demonstrate a direct association between Mig6 and Met (unpublished data). Moreover, the minimal region that was mapped in EGFR to be essential for the binding of Mig6 is not found in the amino acid sequence of mouse Met. However, pull-down experiments with GST-tagged Mig6 confirmed the association with growth factor receptor–bound protein 2 (Grb2) (Fig. 7 A), which had been observed previously (Fiorentino et al., 2000). Control pull-downs with the GST-tagged CRIB domain of P21-associated serine/threonine kinase failed to pull down Grb2 (Fig. 7 A). This suggested the possibility that Mig6 may bind Met indirectly by way of Grb2, thereby inhibiting Met in a receptor-proximal fashion.
Figure 7. The CRIB domain of Mig6 is necessary and sufficient to inhibit HGF-induced cell migration. [A] Mig6 binds Grb2. GST-Mig6 and GST-Mig6\textsuperscript{ΔCRIB} purified fusion proteins were used to pull down endogenous Grb2 from MLP29 total cell lysate. The fusion proteins were incubated for 1 h with total (Tot.) cell lysate and pulled down using glutathione-sepharose beads. Pulled-down proteins were eluted from the beads and analyzed by SDS-PAGE. Western blot (W.B.) analysis using an \(\alpha\)-Grb2 antibody shows that Grb2 associates with Mig6, and that the association is independent of Mig6’s CRIB domain. A GST-PAK\textsuperscript{ΔK} fusion protein was used as a negative control. (B) Mig6 reduces the levels of Cdc42-GTP. The GST-Ack-CRIB–purified fusion protein was used to pull down (P.D.) the active form (GTP-bound) of transfected GFP-Cdc42 at different time points of HGF stimulation in the presence or the absence of Mig6 expression plasmid. MLP29 cells were transfected with GFP-Cdc42 plus GFP (left half) or GFP-Cdc42 plus Mig6 expression plasmids (right half), starved for 48 h in 0.1% FBS, stimulated with 40 ng/ml of HGF for the indicated times, and lysed. The GST-Ack-CRIB protein was incubated with the cell lysates, pulled down using glutathione-sepharose beads, eluted from the beads, and analyzed by SDS-PAGE. Western blot (W.B.) analysis using an anti-GFP–specific antibody showed that exogenous GFP-Cdc42 is activated upon 15 and 30 min of HGF stimulation. The expression of Mig6 significantly reduced exogenous GFP-Cdc42 activation upon HGF stimulation (compare 15- and 30-min time points, left and right part of the blot). Western blot analysis on total cell lysates (TCL) using an anti-GFP–specific antibody was used to control that comparable amounts of total GFP-Cdc42 were used for the pull down. [C] Schematic representation of the different GST-Mig6 deletion mutants. 14-3-3, 14-3-3 binding region; AH, Ack homology domain; PDZ, PDZ target site; SH3BM, SH3 binding site. [D, left blot] GST-Mig6, GST-Mig6\textsuperscript{ΔCRIB}, GST-Mig6\textsuperscript{ΔNT}, and GST-Mig6\textsuperscript{ΔSH3} were transfected in MLP29 cells and pulled down using glutathione-Sepharose beads (Glut.). The proteins were eluted from the beads and analyzed by SDS-PAGE. Western blot (W.B.) analysis using an \(\alpha\)-GST antibody shows that the Mig6 fusion proteins were expressed at similar levels. Transfection with YFP expression plasmid was used as a negative control for the \(\alpha\)-GST antibody. (D, right blot) GST-Mig6 plus GFP-Cdc42, GFP-Cdc42, or GST-Mig6 plus GFP expression plasmids were transfected in MLP29 cells. The cells were lysed, and total cell lysates analyzed by SDS-PAGE. Western blot analysis using an anti-GFP– (top panel) and an anti-GST– (bottom panel) specific–antibody shows that the exogenous Mig6 and Cdc42 (arrowheads) are expressed at equal levels. Transfection of GFP expression plasmid was used as a negative control for the anti-GST and anti-GFP antibodies. Asterisk in panel D denotes an unspecific cross-reactive protein that is detected with anti-GST antibodies.

Quantification of cell migration expressed as fold of induction over unstimulated cells in the presence of 10% FBS (E and G) or HGF (F and H). MLP29 cells were left untransfected or were transfected with the indicated GST-Mig6 expression plasmids (E and F).
We next addressed the relevance of the Mig6 CRIB domain, which had been suggested to bind the Rho family GTPase, Cdc42 (Makkinje et al., 2000). HGF activated Rho family GTPases in epithelial cells concomitant with cell-spreading responses, which was inhibited by dominant negative Cdc42 or Rac (Royal et al., 2000). Mig6 shares striking homology with the noncatalytic region of the cytoplasmic tyrosine kinase, activated Cdc42-associated tyrosine kinase (Ack), which interacts with Cdc42 by way of its CRIB domain and inhibits Cdc42’s GTPase activity (Mott et al., 1999). Using the yeast two-hybrid assay, we first confirmed that full-length Mig6 bound Cdc42 with higher affinity than Rac (Fig. S2). We then asked if Mig6 would prevent the activation of Cdc42 in MLP29 cells. HGF stimulation of MLP29 led to an increase in the levels of GTP-bound Cdc42, as shown by pull-down experiments of transfected Cdc42 using the Ack-CRIB domain (Fig. 7 B). Cotransfection of Cdc42 with Mig6 essentially eliminated this effect (Fig. 7 B). We next investigated if the CRIB domain of Mig6 was essential for Mig6’s inhibition of Cdc42's inhibition of HGF-induced cell migration. We generated GST-tagged versions of Mig6 lacking the CRIB domain; a truncated NH2-terminal fragment of Mig6 including the CRIB domain, but lacking the Ack homology domain; and the isolated CRIB domain only, and expressed them in MLP29 cells (Fig. 7 C). The mutant proteins were expressed at similar levels (Fig. 7 D) and in similar subcellular compartments (Fig. S2).

Next, we analyzed HGF-induced cell migration. Mig6FL inhibited cell migration twofold (Fig. 7 F). In contrast, Mig6 lacking the CRIB domain had no effect on HGF-induced cell migration (Fig. 7 F). The NH2-terminal fragment of Mig6 including the CRIB domain and, more importantly, the isolated CRIB domain inhibited HGF-induced cell migration to the same extent as did Mig6FL (Fig. 7 F). The effects were specific for HGF-stimulated cells, because cell migration that was induced by FBS was not affected by ectopic expression of Mig6 including the CRIB domain, but lacking the Ack homology domain, or CRIB (Fig. 7 E). If the mechanism of Mig6 action involved the binding and inhibition of Cdc42, the coexpression of dominant-active Cdc42 (Cdc42*) may rescue the antimigratory effect of Mig6. To test this, we cotransfected MLP29 cells with Mig6FL and GFP or with the same amounts of Mig6FL and Cdc42* expression plasmids, and performed cell migration assays. The combination of Mig6FL and GFP led to an efficient block of migration, whereas the coexpression of Mig6FL and Cdc42* completely rescued cell migration. Expression of Cdc42* alone had no significant effect in this assay (Fig. 7 H). These results suggest that the CRIB domain of Mig6 is necessary and sufficient for inhibition of HGF-induced cell migration. They further suggest that part of Mig6’s mechanism of action involves the regulation of Rho GTPases, such as Cdc42.

**Discussion**

The complex chain of events that attenuates signal transduction of receptor tyrosine kinases remains poorly understood. Recent studies revealed that negative receptor signaling involves intricate interactions between ubiquitin ligases, adaptor proteins, inhibitory proteins, cytoplasmic kinases, and phosphatases (Dikic and Giordano, 2003). Although some of these negative regulators can act on rather specific targets (protein tyrosine phosphatase inhibits insulin and insulin-like growth factor–1–stimulated signaling), others seem to inhibit multiple receptor tyrosine kinases (e.g., c-Cbl ubiquitin ligase) and generic signaling pathways. In this report, we show that Mig6 is induced by HGF in different cell lineages. Mig6 negatively regulates HGF/Met-mediated cellular responses, including cell migration and neurite growth. This suggests that Mig6 is part of a physiologic mechanism that negatively controls the strength and duration of Met signaling, thereby fine tuning signal transduction. Although further experiments are needed to clarify the exact mechanism of Mig6 action, our data suggest an important role for Mig6 interaction with Rho family GTPases.

**Mig6 induction by external signals**

We identified Mig6 in MLP29 cells as an RNA transcript that was induced highly by HGF and EGF and rather weakly by FGF2 and PDGF. Other investigators found that Mig6 was induced by serum, EGF and related ligands, and cellular stress factors (Wick et al., 1995; Makkinje et al., 2000; and references within). We have provided evidence that Met signaling is a major pathway for Mig6 expression in vivo by showing a reduction of mig6 transcript levels in embryonic liver and lung of mouse mutants expressing a severe signaling hypomorph of Met (Fig. 1). We conclude that in many circumstances, Mig6 is expressed at low levels, and its expression is induced by HGF/Met signaling to activate feedback inhibition with some delay after the initiation of Met signaling. Consequently, the cell responds robustly to Met signaling, until Mig6 levels are high enough to attenuate the Met response. Alternatively, other external signals may have induced the expression of Mig6 before the cells were exposed to HGF, thereby reducing the cell’s ability to respond to Met signaling. Loss of the ability to induce the expression of Mig6 may be part of the multiple step process toward malignancy. Consistent with this model, a recent large-scale expression profiling study noted that Mig6 expression was down-regulated in patients who had breast cancer with short survival time (Amatschek et al., 2004). It seems as if loss of Mig6 provides a growth advantage and perhaps metastatic potential for breast cancer cells.
**Mig6 modulates a variety of cellular responses**

Previous work showed that Mig6 inhibits cell proliferation downstream of ErbB family receptors (Fiorentino et al., 2000; Hackel et al., 2001; Anastasi et al., 2003). This includes studies in which Mig6 was silenced by RNA interference, and provided first evidence for its role as an endogenous inhibitor of EGFR-mediated proliferation (Anastasi et al., 2005; Xu et al., 2005). Until now, the role of Mig6 in ErbB-mediated cell migration had not been addressed. However, this is an important question; numerous studies demonstrated that EGFR and related ligands for EGFR/ErbB receptors stimulate chemotactic migration in vertebrate and invertebrate systems (Wells and Liflin, 2004). Our study has concentrated on the role of Mig6 in Met-mediated cell migration, a process that is implicated in several physiologic contexts, including myoblast migration during development, scattering and branching morphogenesis of epithelial cells, and neuronal migration in the developing forebrain (Powell et al., 2001; Birchmeier et al., 2003; Rosario and Birchmeier, 2003). Met signaling also is critical for neurite extension and branching of different neuronal subpopulations (Maina and Klein, 1999; Thompson et al., 2004), a process that has similarities with invasive growth of malignant cells (Trusolino and Comoglio, 2002). We found that Mig6 overexpression effectively and specifically reduced HGF-induced migration of a cell line of hepatic origin and of primary cortical neurons. Mig6 also effectively blocked HGF-induced neurite growth of primary sympathetic neurons. In converse experiments, mig6 knock-down effectively enhanced cell migration of hepatic progenitor cells and mildly, yet significantly, enhanced neurite growth of sympathetic neurons.

**Mig6 acts distally from Met by way of interaction with Rho family GTPases**

Mig6 is a multidomain molecule whose amino-terminal 38 amino acid residues show homology with the conserved CRIB domain that is present in a variety of intracellular signaling molecules (Pirone et al., 2001). Mig6 contains putative binding sites for SH3-containing molecules such as Grb2, PI3K, and PLCγ (Fiorentino et al., 2000), and for 14–3–3 and PDZ domain containing proteins. Notably, Mig6 includes a COOH-terminal region that is highly homologous to the non-catalytic portion of Ack1. A region within this Ack1 homology domain was identified as an EGFR-binding motif (Fiorentino et al., 2000; Anastasi et al., 2003) that was necessary and sufficient for the inhibition of EGFR signaling (Xu et al., 2005). Expression of Mig6/Receptor-associated late transducer in tumor cells modestly reduced the levels of ErbB-mediated phospho-MAPK and phospho-Akt expression, which suggested some interference with mitogenic signaling pathways (Anastasi et al., 2005). Conversely, loss of Mig6 led to sustained MAPK phosphorylation in EGF-stimulated keratinocytes (Ballaro et al., 2005 and unpublished data). The association between Mig6 and Met by way of Grb2 interaction (Fig. 7) suggests the possibility that Mig6 inhibits Met signaling in a receptor-proximal fashion. However, we have been unable to detect changes in the levels of Met-mediated phospho-MAPK and phospho-Akt expression in cells that transiently overexpress Mig6 (Fig. S2).

Therefore, we favor the view that the antimigratory effect of Mig6 in MLP29 cells involves other pathways. Because the CRIB domain of Mig6 binds Cdc42 in a GTP-dependent manner (Makkinje et al., 2000 and this report), HGF stimulation activates Cdc42, and activated Cdc42 is required for HGF-induced lamellipodia formation and cell movement (Royal et al., 2000), we favor the possibility that Mig6 inhibits Cdc42-mediated cell movement by way of its CRIB domain. In support of this hypothesis, we show that (i) overexpression of a Mig6 construct lacking the CRIB domain, but retaining Grb2-binding capabilities, is unable to suppress HGF-induced migration; (ii) overexpression of the Mig6 CRIB domain alone is sufficient to inhibit HGF-induced migration; and (iii) coexpression of Mig6 with a dominant-active form of Cdc42 rescues the antimigratory effects of Mig6. Therefore, the mechanism of Mig6 inhibition of Met resembles the mechanism of Ack1 inhibition of EGFR. The Caenorhabditis elegans orthologue of Ack1, Ark, associates with EGFR by way of binding to Sem5, the C. elegans orthologue of Grb2 (for review see Worby and Margolis, 2000). Similar to Mig6, overexpression of the CRIB domain of Ack1 is sufficient to inhibit growth factor–induced activation of Cdc42 (Nur-E-Kamal et al., 1999). Furthermore, overexpression of the CRIB domain of another small GTPase-binding protein, PAK (an effector of Rac1), by way of its interaction with Rac1, inhibits Semaphorin 3A-induced growth cone collapse (Vastrik et al., 1999). Together, these results indicate that CRIB domains are sufficient to inhibit the biologic effects of specific GTPases.

We propose that Mig6 is part of a network of negative signaling molecules that fine tune and attenuate Met and ErbB signaling in development and disease. The analysis of mig6 mutant mice provided genetic evidence for a role of Mig6 in the maintenance of joints and cartilage (Zhang et al., 2005) and in skin morphogenesis and cancer (unpublished data). They will be an invaluable tool in our efforts to elucidate the inhibitory functions and molecular interactions of Mig6 in the context of an intact tissue.

**Materials and methods**

**Mouse 15,000 cDNA microarray production and data analysis**

The “NIA mouse 15k cDNA microarray” chip production, isolation of RNA, labeling of cDNA probes, and hybridization to the cDNA array was performed as described (Cortes-Cantell et al., 2004). In brief, the hybridization was performed in duplicate using total RNA samples that were extracted from two independent batches of cultured cells. Stimulated and mock-treated samples were labeled by Cy5 and Cy3-dUTP, respectively. Data acquisition and initial data analysis were performed with GenePix Pro 3.0, and data analysis were analyzed further in Microsoft Excel to obtain the gene list. Quality control was performed by eye to confirm scanner alignment and absence of significant bubbles and scratches. Scatter plots were used further to eliminate the unacceptable hybridization data. GenePix Pro program calculates the normalization factor of each hybridization, based on the premise that the arithmetic mean of the ratios from every feature on the given array should be equal to 1. Therefore, normalization was performed by multiplying the factor to ratio of medians (ROM) in each gene. The genes that passed all of these criteria were sorted first by sum of median (SOM), which indicates the intensity of hybridization. To obtain the list of genes that are relatively abundant, the genes that showed more than 5,000 of SOM were selected. Finally, the genes were sorted by ROM. The genes that showed more than ±1.5-fold changes were selected in the data table from each hybridization. Finally, the data were compared between the two hybridizations and average ROM was calculated. Gene functions were categorized...
Based on the information given in the NIH mouse 15k cDNA clone gene ID list at the first instance and modified when necessary.

**Northern blots**

Total RNA was extracted from different cell lines in various experimental conditions using the RNeasy Clean Solution (Qiagen). 20 μg total RNA was electroporated and blotted onto Genescreen nylon membrane (NEN Life Science Products). Labeled probes were generated using random primers and hybridized (6× SSC, 5× Denhardt solution, and 100 μg salmon sperm DNA) with the membrane for 18 h at 65°C. cDNA inserts used as probes were obtained by NotI/Sall double restriction digestion of the NIH mouse 15k cDNA clones.

**RNA interference**

siRNA oligonucleotides (first set [AAGGUCAAGCUUGCCCCCUC-dTdT] and second set [GAGGCAUAGUAUGUGDG-dTdT]) were designed and used for cell transfections as described (Elbashir et al., 2001). Sense and anti-sense siRNA oligonucleotides (DARMAKOM) were diluted in annealing buffer (100 mM K-Acetate, 30 mM Hepes-KOH, 2 mM Mg-Acetate) to the final concentration of 20 μM, denatured for 1 min at 90°C, and annealed by incubation for 1 h at 37°C. 6 μl siRNA duplex was transfected into 4 × 10⁵ cells using oligofectamine (Invitrogen), according to the manufacturer’s instructions. After transfection, cells were left in Dulbecco’s minimum essential medium (DMEM) plus 0.1% FBS for 72 h and transfected a second time with the same siRNA duplex. The aphidicolin treatment was performed as described above. 24 h after the second transfection, cells were harvested and plated on the upper face of the Boyden chamber as described above.

**Plasmids and expression vectors**

Primer sequences are available upon request. The V5 COOH-terminal, GST NH₂-terminal, and His COOH-Terminal tagged Mig6 full-length and mutant proteins were generated using the Gateway Cloning technology according to the manufacturer’s instructions (Invitrogen). In brief, Mig6 full-length and deletion mutants were amplified by PCR from the IRAK clone IRAKp691F0910 using oligonucleotides that contained the minimal recombination sequences (5′-ATB and 3′-ATB). PCR amplified products were excised using pDONOR201 vector by the use of GT110201 and transformed in the manufacturer. The pDONOR201 vectors containing Mig6 full-length or deletion mutants were shuttled into pCDNA 6.2 COOH-Terminal-V5, pDEST 27 NH₂-terminal-GST, and pDEST26 NH₂-terminal-His plasmids (all Invitrogen) for mammalian expression or into pDest15-N-Herm-GST for bacterial expression. The LacZ/V5 expression plasmid was purchased (Invitrogen). GFP-Cdc42 (Yoshida and Akiyama, 2000) and dominant active expression plasmids were provided by M. Way. The CIB domain of Ack was amplified by PCR from the I.M.A.G.E. Consortium cDNA Clones (Clone ID IMAGG9991101392Q3) (Lennon et al., 1996) and was subcloned into pCRII-TOPO cloning vector (Invitrogen) following the manufacturer’s instructions. The fragment was inserted into the pGEX-F vector for bacterial expression. The pGEX-NH₂-terminal-GST-Mig6, containing the Mig6 COOH-Terminal half (from aa 273 to 459), was provided by A. Ullrich and was used to produce the Mig6 antigen.

**Primary cortical neuron culture, cell migration assays, and growth factors**

MLP29 cells were cultured and transiently transfected as described previously (Muller et al., 2002). C2C12 were grown as described (Yaffe and Saxel, 1977). Primary hepatocytes were cultured as described (Maina et al., 2001). The transwell assay was performed as described previously (de Luca et al., 1999). In brief, 10⁵ MLP29 cells were seeded on the upper face of the Boyden chamber membrane (8 μm pore; Costar), which was coated previously with 0.15 μg/cm² of fibrinogen (Sigma-Aldrich). The cells were stimulated with 10 ng/ml of hepatocyte growth factor (HGF) (Molecular Probes) and 10 ng/ml insulin for 24 h before and during the migration assay with DMSO-(Fluka) or 1.6 μg/ml aphidicolin-containing media (Sigma-Aldrich).

Cortical neurons were obtained by digestion of E15.5 mouse telencephalon in 1× PBS containing trypsin (EDTA7°C, Gibco BRL). The neurons were washed twice with DMEM-F12 supplemented with 10% horse serum (GIBCO BRL), washed once in neurobasal medium containing B27 supplement (NB/B27, 50:1, GIBCO BRL) and were dissociated with a fire-polished glass Pasteur pipette. Cortical neurons were dissected and electroplated by mixing 6 × 10⁵ cells with 24 μg expression plasmid, transferred into an electroporation cuvette (MBP Molecular Bioproducts), and electroporated (five pulses at 270 V of 3 msec separated by 1-s inter-

**Antibodies**

The rabbit anti-Mig6 antibody was generated as described previously (Hackett et al., 2001). The anti-phospho-Mapk, anti-MAPK, and anti-phospho-Akt (New England Biolabs, Inc.) antibodies were used as described previously (Maina et al., 2001). Antibody dilutions: monoclonal anti-tubulin (Sigma-Aldrich) 1:2,000 for Western blot (WB) analysis; monoclonal anti-V5 (Invitrogen) 1:1,000 and 1:250 for WB and immunocytochemical (IC) analysis, respectively; rabbit polyclonal anti-Met (Biomol Research Laboratories) and anti-GST (Santa Cruz Biotechnology, Inc.) 1:500 for WB analysis; anti-microtubule-associated protein 2 (Sigma-Aldrich) 1:10,000 for IC analysis; monoclonal anti-Grob2 (Transduction Labs) 1:1,000 for WB analysis; donkey polyclonal anti-mouse Alexa488 (Molecular Probes) and anti–rabbit CY3 (Jackson ImmunoResearch Laboratories) 1:200 for IC analysis; and polyclonal goat anti-mouse and anti–rabbit HRP-conjugated (GE Healthcare) 1:2,000 for WB analysis.

**Imageing**

All cell images were obtained with an Axioplan-2 imaging fluorescent microscope (Carl Zeiss Microimaging, Inc.) equipped with a RT Slider 2.3.1 digital color camera (Diagnostic Instruments). A 40× objective was used, except for the in situ hybridization images that were taken with a 20× lens (Carl Zeiss Microimaging, Inc.).

**Yeast two-hybrid system**

The NH₂-terminal half of Mig6 and the CIB domain of PAK and Ack were inserted by way of BamH/I into the yeast two-hybrid prey vector pAct2. Cdc42 and Rac were inserted by way of BamH/I/Sall into the pAct2 vector. These plasmids were transformed in the indicated combination into the reporter strain PJ69-4A according to the CLON-TECH Laboratories yeast protocol handbook. The double transformants were selected by plating onto synthetic media lacking leucine and tryptophan with 2% glucose as the carbon source (Leu⁻/Trp⁻). Double transformants were restreaked onto Leu⁻/Trp⁻ or synthetic media lacking leucine, tryptophan, histidine, and adenine (quadruple drop out).

**Recombinant protein purification and Cdc42 activation assay**

Recombinant GST fusion proteins were purified according to standard procedures. MLP29 cell lysates were incubated for 1 h at 4°C with recombinant proteins immobilized on glutathione-sepharose. The glutathione-sepharose beads were washed several times in cell lysis buffer, eluted with sample buffer, and analyzed by immunoblotting. For the Cdc42 activation assay, MLP29 cells in various experimental conditions were lysed (50 mM Tris-Cl pH 7.4, 5 mM MgCl₂, 200 mM NaCl, 1 mM sodium orthovanadate, 1% NP-40, 10% glycerol, and a mixture of protease inhibitors EDTA-free), and the lysates were incubated with the recombinant GST-Ack/CRIB protein bound to glutathione-sepharose beads for 1.5 h at 4°C. The proteins were eluted from the beads, and analyzed by SDS-PAGE and immunoblotting.

**Paravertebral sympathetic neuron cultures**

Superior cervical ganglia were dissected from postnatal day 40 (P40) CD1 mice. Neuron cultures were set up as described (Thompson et al., 2004). HGF stimulation and quantification of neuronal survival was done...
as described (Thompson et al., 2004). Sholl analysis was performed as described previously (Sholl, 1953). For protein extraction, primary cultures of dissociated P40 superior cervical ganglion neurons were grown for 3–6 h in culture before being stimulated for different times with HGF. Cells were harvested and homogenized as described above.

Online supplemental material
Fig. S1 shows Northern blot analysis of selected genes from the gene list. Fig. S2 shows the effects of Mig6 on canonical Met signaling, yeast two-hybrid interactions between Mig6 and Cdc42/Rac, and immunohistochemical analysis of overexpressed and endogenous Mig6. Table S1 and Table S2 list the genes that are regulated by HGF in MLP29 cells. Online supplemental material available at http://www.jcb.org/cgi/content/full/jcb.200502013/DC1.

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