Regulation of Integrin Activity by MIA*

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MIA (melanoma inhibitory activity) has been identified as a small protein secreted from malignant melanoma cells, which interacts with extracellular matrix proteins including fibronectin. Here, we show that MIA negatively regulates the activity of the mitogen-activated protein kinase pathway in malignant melanoma. Using far Western blotting and co-immunoprecipitation we searched for MIA-binding cell surface proteins. We found that MIA interacts with integrin α4β1 and α5β1, leading to down-regulation of integrin activity and reduction of mitogen-activated protein kinase signaling. These findings also suggest that MIA may play a role in tumor progression and the spread of malignant melanomas via mediating detachment of cells from extracellular matrix molecules by modulating integrin activity. Inhibiting MIA functions in vivo may therefore provide a novel therapeutic strategy for metastatic melanoma disease.

We have previously identified MIA (melanoma inhibitory activity), an 11-kDa protein secreted into the tissue culture supernatant from malignant melanoma cells (1). MIA expression in vivo correlates with progressive malignancy of melanocytic tumors (2). Additionally, in recent studies we detected enhanced MIA protein levels specifically in the serum of patients with metastatic melanomas (3). In vitro studies revealed a role for MIA in supporting the invasive and migratory potential of melanoma cells. In vivo studies in two animal model systems confirmed the importance of MIA in melanoma metastasis. MIA expression levels parallel closely the capability of melanoma cells to form metastases in syngeneic animals (4, 5).

Three-dimensional analyses of MIA by multidimensional NMR (6–8) or x-ray crystallography (9) indicate that MIA defines a novel family of secreted proteins that adopt an SH3 domain-like fold in solution. Furthermore, NMR spectra revealed that MIA interacts with peptides matching to type III human fibronectin repeats that are closely related to α4β1 integrin-binding sites (6). These data support a model in which MIA regulates attachment to specific components of the extracellular matrix. Based on these results and on the observation that MIA alters melanoma cell morphology, we determined that MIA treatment results in cell detachment by decreasing interactions between melanoma cells and extracellular matrix molecules (10). The study presented here was performed to find additional MIA-interacting proteins and to identify signaling pathways regulated by MIA.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—The melanoma cell lines Mel Im and Mel Ei have been described in detail previously (11). The cell line Mel Ei was derived from a primary cutaneous melanoma, and the cell line Mel Im was derived from a metastasis of malignant melanoma. To establish fibronectin-deficient fibroblast-like cells, primary embryonic fibroblasts were isolated from E13.5 fibronectin (floX/floX) embryos and immortalized by retroviral transduction of the SV-40 large T antigen and cloned (12). Subsequently, two clonal lines were treated with a cre-transducing adenovirus to delete the floxed fibronectin genes. The deletion of fibronectin alleles in both cre-treated clones was confirmed by PCR, and the lack of Fn protein expression was confirmed by immunoprecipitation using metabolically labeled conditioned media of these cells. Integrin β1-deficient cells were generated as previously published (13).

For tissue culture the cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with penicillin (400 units/ml), streptomycin (50 μg/ml), 1-glutamine (300 μg/ml), and 10% fetal calf serum (Sigma) and split 1:5 every 3 days. Normal human epidermal melanocytes and keratinocytes derived from normal skin were cultivated in melanocyte medium MGM-3 or keratinocyte growth medium (Promocell, Heidelberg, Germany), respectively, under a humidified atmosphere of 5% CO2 at 37 °C.

Expression and Purification of Recombinant MIA Protein—Escherichia coli M15(pREP4) cells transfected with the expression plasmid pQE40-MIA expressing 108 residues of human MIA (Gly25–Gln131) were grown to an absorbance A600nm = 0.6, induced by 1 mM isopropyl-1-thio-β-D-galactopyranoside for 4 h and lysed by sonication. The protein was refolded from E. coli inclusion bodies as previously published (1). Refolded human MIA was applied to hydrophobic interaction chromatography and further purified on a S-Sepharose Fast Flow (Sigma). Finally, gel filtration was performed on a Superdex 200 Prep Grade (Sigma). Fractions containing human MIA were pooled and concentrated. Purified protein was checked by SDS-PAGE and high pressure liquid chromatography and was shown to be 95% pure (data not shown).

Expression of Recombinant Biotinylated MIA Protein—A MIA prokaryotic expression vector with a 15-amino acid Avi tag peptide sequence including a Fxa cleavage site was constructed by overlap extension PCR. The MIA cDNA construct was cloned into the vector pLVE2X2.3-MCS (Roche Applied Science). The expression vector was used in the rapid translation system, a cell-free E. coli-based protein transcription/translation system (Roche Applied Science). The expression vector was used in the rapid translation system, a cell-free E. coli-based protein transcription/translation system (Roche Applied Science). By adding biotin, ATP, and the E. coli biotin protein ligase BirA during the procedure, the protein was biotinylated at the introduced Avi tag at the N terminus and called biotinylated MIA. The correct function and folding of the protein was tested by performing a well established MIA-ELISA2 and functional assays.

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2 The abbreviations used are: ELISA, enzyme-linked immunosorbent assay; MAP, mitogen-activated protein; MAPK, MAP kinase; PBS, phosphate-buffered saline; BSA, bovine serum albumin; RIPA, radioimmune precipitation assay; PVD, polyvinylidene difluoride; FACS, fluorescence-activated cell sorter; ERK, extracellular signal-regulated kinase.
**MIA Regulates Integrin Activity**

**Assays for Protein-Protein Interaction—**Purified integrins (α4β1 (1:100 in PBS), α5β1 (1:500 in PBS) (14)), 5% BSA in PBS, as control, or biotinylated MIA (1 µg/ml in PBS), respectively, were coated each in a volume of 100 µl onto 96-well high binding protein plates (Costar) overnight at 4 °C. After 17 h the coated wells were blocked with 200 µl of blocking buffer (25 mM Tris-HCl, 150 mM NaCl, and 5% BSA) for 2 h, washed three times with 200 µl of binding buffer (150 mM NaCl, 25 mM Tris-HCl, 1 mM MnCl2, and 0.1% BSA), and exposed to 100 µl of 1 µg/ml purified biotinylated MIA in binding buffer for 2 h at room temperature. The controls were exposed to 1 µg/ml BSA. After three washing steps with binding buffer, MIA binding was quantified using a peroxidase-coupled monoclonal anti-MIA antibody and the substrate 2,2'-azino-di[3-ethylbenzthiazoline sulfonate] (ABTS) (Roche Applied Science). The reactions were measured at A405 nm. The experiments were repeated three times with similar results.

For co-immunoprecipitation 100 µg of cell lysates dissolved in binding buffer (20 mM NaPO4, 150 mM NaCl, pH 7.5) were precleared with 25 µl of protein streptavidin-coupled G-Sepharose (Amersham Biosciences) at 4 °C overnight. After centrifugation the supernatant was transferred into a fresh vial and incubated with biotinylated MIA with shaking at 4 °C overnight. Then 50 µl of protein streptavidin-coupled G-Sepharose was added for 1 h, pelleted, washed three times with binding buffer, resuspended in 20 µl of Laemmli’s buffer, heated at 95 °C for 5 min, and separated on 12% SDS-polyacrylamide gels. All of the proteins binding to MIA were stained by silver staining (Silver Express Staining; Invitrogen). The experiments were repeated three times with similar results. Additionally, specific proteins were identified by Western blot.

**Protein Analysis in Vitro (Western Blotting)—**For protein isolation 2 × 10⁶ cells were washed with 1× PBS and lysed in 200 µl of RIPA buffer (Roche Applied Science). The protein concentration was determined using the BCA protein assay reagent (Pierce). Balanced amounts of cell proteins (50 µg) were denatured at 70 °C for 10 min after addition of Roti-load-buffer (Roth, Karlruhe, Germany) and subsequently separated on NuPAGE-SDS gels (Invitrogen). After transferring the proteins onto PVDF membranes (Bio-Rad), the membranes were blocked in 3% BSA/PBS for 1 h and incubated with a 1:1000 dilution of primary polyclonal rabbit anti integrin α2, 3, 4, or 5 antibody or anti-ERK1/2, anti P-ERK1/2 (R & D, Richmond, VA; Chemicon, Hampshire, UK) overnight at 4 °C. A 1:2000 dilution of anti-rabbit-AP (Chemicon) was used as a secondary antibody. Staining was performed using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate solution (Zytomed, Heidelberg, Germany). All of the experiments were repeated at least three times with similar results. The peptide spot analysis (Jerini, Berlin, Germany) was performed to assay kinase activity following the manufacturer’s instructions.

**MIA Far Western—**SDS-PAGE was performed with 40 µg of RIPA lysates from melanoma cell lines and controls, respectively. The proteins were transferred to PVDF membrane by electroblotting. The membrane was blocked with 3% BSA for 30 min and incubated with biotinylated MIA (1 µg/ml) for 3 h at 4 °C. After three washing steps with PBS, the membrane was incubated with alkaline phosphatase-coupled streptavidin for 30 min. Staining was performed using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate solution (Zytomed). The experiments were repeated three times with similar results.

**MIA-ELISA—**MIA produced recombinantly was measured using an ELISA system (Roche Applied Science). 1 × 10⁶ cells were cultured in 5 ml of serum-free Dulbecco’s modified Eagle’s medium, and the amount of MIA secreted into the supernatant was quantified by ELISA. Monoclonal antibodies coupled with biotin or peroxidase, respectively, were used to quantify MIA in a 96-well plate coated with streptavidin. 2,2’-Azino-di[3-ethylbenzthiazoline sulfonate] (ABTS) was used as substrate and quantified at A405 nm.

**FACS Analysis—**To analyze binding of MIA to the cell surface, the cells were detached from flasks using 5 mM EDTA and resuspended in PBS. 10⁶ cells were incubated with 1 µg/ml biotinylated MIA for 1 h at 4 °C. For specific competition probes were incubated with 400 and 800 ng/ml recombinant MIA, respectively. After three washing steps with PBS, the secondary antibody (neutavidin/fluorescein isothiocyanate) was added for 1 h at 4 °C. After additional washing with PBS, FACS analysis was performed.

To analyze whether MIA regulates integrin activity, the cells were detached from flasks using 5 mM EDTA in PBS. The cells were resuspended in PBS (10² cells/ml) containing 4.5 g/liter glucose and treated with biotinylated MIA (1 µg/ml), with MnCl₂ or with EDTA. One mM MnCl₂ was used to activate all integrins, and 1 mM EDTA was used to inactivate integrin activity (15). 50 µl of the treated cells were mixed with 50 µl of the primary antibody 12G10 (20 µg/ml) (specific for activated integrins) and incubated for 60 min on ice. Additionally, an antibody was used staining β1 independent on the activation status (Chemicon). After three washing steps with PBS with 1% fetal calf serum, the cells were stained with the secondary antibody streptavidin/cy5 in PBS with 10% serum for 30 min. After three washing steps the probes were fixed (100 µl of PBS, 100 µl of 2% formaldehyde), washed, and resuspended in a volume of 300 µl of PBS. FACS data were analyzed using the CellQuest software (Becton Dickinson). Statistical significance was determined by Kolmogorov-Smirnov analysis. All of the experiments were repeated three times.

**Statistical Analysis—**The results are expressed as the means ± S.D. (range) or percentages. Comparison between groups was made using the Student’s paired t test. A p value < 0.05 was considered statistically significant. All of the calculations were performed using the GraphPad Prism software (GraphPad Software Inc, San Diego, CA).

**RESULTS**

**MIA Reduces MAP Kinase Activity in Malignant Melanoma—**One aim of this study was to identify signaling pathways regulated by MIA. To analyze the influence of MIA treatment on MAP kinase (MAPK) activity, we used an assay measuring phosphorylation of myosin-binding protein by MAPK. This activity assay revealed a strong reduction of MAPK activity with a maximum 1 h after the beginning of MIA treatment (Fig. 1A). After excluding the MAPKs p38 and c-Jun N-terminal kinase to be regulated via MIA (data not shown), we used antibodies against phosphorylated ERK1/2 to specifically analyze ERK activity after MIA treatment. MIA treatment induced a strong reduction of ERK1/2 activity in melanoma cell lines; maximum of inhibition was detected 45 min to 2 h after beginning of MIA treatment (Fig. 1B). Regulation of ERK activity by MIA was confirmed by peptide spot analysis (data not shown).

MIA is known to bind to several matrix proteins including fibronectin and to promote detachment of melanoma cells (10). To investigate whether the reduction of ERK1/2 phosphorylation depends on MIA binding to fibronectin and consecutive detachment of the cells, we treated melanoma cells with RGD peptides. The RGD sequence in protein domains is known to be a binding site for integrins in extracellular matrix proteins like fibronectin and to be crucial for cells to perceive and attach to their environment. Only a weak reduction of ERK1/2 phosphorylation was detectable after treating the cells with RGD peptides.
which implies that MIA influences ERK1/2 activity at least additionally via a different cellular surface motif than the RGD sequence (Fig. 1C).

MIA Binds to Cell Surface—To identify potential cell surface receptors and modulators of cell signaling, we used biotinylated MIA to analyze binding of MIA to the cell surface. FACS analysis revealed specific binding of MIA to the cell surface of melanoma cells (Fig. 2). The values in percentages give the results of the densitometric analysis. All of the assays were repeated at least three times, and representative results are presented.

FIGURE 2. FACS analysis of MIA binding to the cell surface of melanoma cells. All of the results are presented as dot plots. A, Mel Im cells incubated with streptavidin/Cy5 without addition of biotinylated MIA were analyzed by FACS. B, Mel Im cells incubated with biotinylated MIA (1 μg/ml) and streptavidin/Cy5 were analyzed by FACS. Binding of MIA to the cell surface of the melanoma cell line Mel Im was detected. C and D, Mel Im cells incubated with biotinylated MIA (1 μg/ml) followed by incubation with unlabeled MIA (400 ng/ml (C) and 800 ng/ml (D)) and streptavidin/Cy5 were analyzed by FACS. Competition of biotinylated MIA with unlabeled MIA on Mel Im cells revealed that MIA binding to the surface is specific. E, mouse fibroblasts incubated with streptavidin/Cy5 without the addition of biotinylated MIA were analyzed by FACS. F and G, cell binding of MIA to mouse fibroblasts that are fibronectin-negative (F) or positive (G), respectively, was analyzed by FACS. The shift in fluorescence did not differ between the two cell lines, indicating that MIA binding is not modulated by fibronectin. All of the assays were repeated at least three times, and representative results are presented.

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FIGURE 1. Influence of MIA on MAP kinase activity in melanoma cells. Melanoma cells were incubated with MIA for different periods of time, and subsequently, cell lysates were generated. A, equal amounts of cell lysates were incubated with myosin-binding protein (MBP) to analyze MAP kinase activity, and phosphorylated myosin-binding protein was subsequently detected by Western blot. Down-regulation of MAP kinase activity by MIA was detected. Maximum of inhibition was observed after 1 and 2 h. B, Western blot analysis of ERK phosphorylation in Mel Im cell lysates revealed a decrease in ERK phosphorylation with a maximum of inhibition after 45 min to 2 h. To ensure equal loading, ERK1/2 was stained as a control. C, incubation of melanoma cells with RGD peptides for different periods of time revealed no significant changes in ERK phosphorylation. To ensure equal loading, ERK1/2 was stained as a control. The values in percentages give the results of the densitometric analysis. All of the assays were repeated at least three times, and representative results are presented.
detectable \( (p < 0.001; \text{Fig. 2B}) \) as compared with the negative control (Fig. 2A). This shift could be competed by unlabeled MIA protein, indicating the specificity of the binding of MIA to the cell surface (400 ng/ml unlabeled MIA (Fig. 2C) and 800 ng/ml (Fig. 2D) compared with Fig. 2B). Quantitative measurements showed complete competition of the signals on the cell surface. Because MIA is known to bind to fibronectin, we used mouse embryonic fibroblasts with or without fibronectin expression in the same experiment. Here, FACS analysis revealed no differences in binding of MIA between the two cell lines (Fig. 2E and 2F), indicating that binding of MIA to the cell surface was not mediated via fibronectin. Fig. 2E shows the negative control for the mouse fibroblasts.

Identification of MIA Binding Partners—To identify MIA-binding proteins, co-immunoprecipitation (Fig. 3A) and far Western blot analysis (Fig. 3B) were performed. Several interacting proteins were found. Interestingly, both assays revealed bands of \( \sim 70, 80, \) and 100 kDa, respectively (Fig. 3A and B). In co-immunoprecipitation assays, binding of these proteins could be almost completely competed by an access

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**FIGURE 3.** Characterization of MIA binding partners. A, co-immunoprecipitations were performed using biotinylated MIA protein and total protein lysates of the melanoma cell line Mel Im. Subsequent separation on SDS-PAGE and silver staining revealed three bands at \( \sim 80 \) kDa \(+/−\) biot. MIA, which could be almost completely competed with equivalent amounts of recombinant MIA \(+/−\) MIA. As control (negative control), melanoma cell lysates were co-immunoprecipitated without the addition of biotinylated MIA. B, far Western blot labeled with biotinylated MIA revealed bands at \( \sim 70−80 \) kDa (first and second lanes) in contrast to the negative control (third lane). The RIPA lysates were separated on SDS-PAGE, blotted onto a PVDF membrane, and incubated with biotinylated MIA followed by streptavidin/alkaline phosphatase. Staining was performed via nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate. All of the assays were repeated three times, and representative results are presented.

**FIGURE 4.** Integrins \( \alpha4 \) and \( \alpha5 \) immunoprecipitate with biotinylated MIA. Cell lysates of normal human epidermal melanocytes (NHEM) and melanoma cells (Mel Im and Mel Ei) were co-immunoprecipitated with biotinylated MIA \(+/−\) MIA. Subsequently, they were separated by SDS-PAGE, blotted onto a PVDF membrane, and immunolabeled with anti-integrin \( \alpha4 \) and \( \alpha5 \) antibodies. The experiment revealed binding of MIA to \( \alpha5 \) (A) and \( \alpha4 \) (B) in integrin. As control melanoma cell lysates were co-immunoprecipitated without the addition of biotinylated MIA (Mel Im). C, Western blot of cell lysates from melanocytes and Mel Im melanoma cell line with anti-integrin \( \alpha4 \) and \( \alpha5 \) antibodies. The experiment revealed binding of MIA to \( \alpha5 \) (A) and \( \alpha4 \) (B). Staining with anti-integrin \( \alpha5 \) antibody showed the larger, 130-kDa form in melanocytes. In the melanoma cell line Mel Im, both the 130-kDa and the processed forms appeared.

**FIGURE 5.** MIA binds directly to integrins \( \alpha4 \) and \( \alpha5 \). ELISA demonstrated binding of biotinylated MIA to integrins \( \alpha4 \) (A) and \( \alpha5 \) (B), respectively. These interactions were competed in a stepwise manner by the addition of soluble integrins in two different concentrations (third and fourth columns), and prevented by addition of EDTA in two concentrations (fifth and sixth columns). The experiments were repeated four times.
of unlabeled MIA (Fig. 3A). Isoforms of integrins of these sizes were reported in several publications (16–18). Therefore, MIA co-immunoprecipitated melanoma cell lysates were analyzed in Western blots to determine whether the bands detected by far Western blot analysis (Fig. 3B) and co-immunoprecipitation (Fig. 3A) resemble integrins. Antibodies against α2 and α3 did not show cross-reactivity with the immunoprecipitated proteins (data not shown). However, anti-α4 and -α5 antibodies revealed specific staining (Fig. 4, A and B). Fig. 4 (A and B) shows specific immunolabeling for α4 and α5 after MIA co-immunoprecipitation of RIPA lysates of melanocytes and of the melanoma cell lines Mel Im and Mel Ei. In the control without biotinylated MIA, no staining was found. In accordance with the α4-stained immunoblot of cell lysates (Fig. 4C), a band occurred at ~70 kDa and at 80 kDa, respectively (Fig. 4B). In immunoblots labeled with the α5 antibody, the unprocessed form of α5 appeared at ~130 kDa in melanocytes, whereas in the melanoma cell line Mel Im smaller bands were detectable at ~80 kDa (Fig. 4C). All of the RIPA lysates that were immunoprecipitated with biotinylated MIA clearly showed bands at ~80 kDa.

MIA Binds Directly to Integrin α4β1 and α5β1—To confirm direct binding of MIA to α4 and α5 integrins an ELISA with plates coated with the respective integrins was applied. MIA binding to both α4 (Fig. 5A) and α5 (Fig. 5B), integrin was demonstrated. Soluble α4 (Fig. 5A) and α5 (Fig. 5B) proteins competed MIA binding in a dose-dependent manner, indicating the specificity of the interaction between MIA and α4 and α5, respectively. Furthermore, we denatured the integrins bound to the plate by incubating them with 1 and 5 mM EDTA, respectively. EDTA complexes divalent cations and thus hinders integrins to establish their active conformation (19). Under these experimental conditions, MIA

FIGURE 6. MIA inhibits integrin activity. FACS experiments using the melanoma cell line Mel Im and the antibody 12G10, which is specific for activated integrins, were performed. A, dotted line, activated integrins after treatment with Mn²⁺; dashed line, Mel Im cells only treated with 12G10; bold line, Mel Im cells incubated with biotinylated MIA and Mn²⁺. The black line resembles Mel Im cells without treatment. B, experiment as in A, but an activation independent antibody anti-β1 was used. Both assays were repeated three times, and representative results are presented.

FIGURE 7. RGD does not compete with MIA for integrin binding. The influence of RGD peptides on MIA binding to α4β1 (first through fourth columns) and α5β1 (fifth through eighth columns) was analyzed by ELISA assays and competition with RGD peptides. Different concentrations of RGD peptide do not compete with MIA on α4 (second through fourth columns compared with first column) or α5 (sixth through eighth columns compared with fifth column). The experiment was repeated three times.
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binding was completely inhibited, indicating that MIA does not bind to
inactivated integrins (Fig. 5).

MIA Inhibits Integrin Activity—Because MIA can inhibit MAPK activity,
and integrins are known to modulate MAPK signaling (19), we specu-
lated whether MIA inhibits integrin signaling by binding to integrin het-
erodimers. To analyze a putative effect of MIA on activated integrins, we
performed FACS assays using the antibody 12G10, which is specific for
activated integrins. Preparation of the cells for the FACS experiment
resulted in inactive integrins (Fig. 6A, dashed line). Therefore, integrin
activation was induced by pretreatment with 1 mM MnCl2 (Fig. 6A, dotted line).
Subsequent MIA incubation significantly down-regulated integrin activity
\( p < 0.001 \) (Fig. 6A, bold line), almost reaching the level before MnCl2
activation. In contrast, when cells were pretreated with EDTA to inhibit
integrin activity, no effect of MIA was detected (data not shown). As control
we performed additional ELISA experiments. Consistent with our data
in the equivalent experiment was performed using an antibody detecting inte-
grin activity, no effect of MIA was detected (data not shown). As control
for integrin activation, we did not find any competition of biotinylated MIA
peted with MIA for binding to H/120 (Fig. 9A). The same finding was observed
for integrin \( \alpha 4 \beta 1 \); however, competition was not as strong (Fig. 9A).
In contrast, the H/120 fragment did not compete with MIA binding to
\( \alpha 5 \beta 1 \) (Fig. 9B). At a concentration of 5 \( \mu g/ml \) H/120 even a slightly
supporting effect of MIA binding to both integrins \( \alpha 4 \beta 1 \) and \( \alpha 5 \beta 1 \) was
revealed (Fig. 9B).

In the reversal of the experiment (Fig. 9C), where the fibronectin
fragments were coated to the plates, \( \alpha 5 \beta 1 \) competed with MIA for
binding to the 50K fragment in a dose-dependent manner, whereas
\( \alpha 4 \beta 1 \) competed only weakly with MIA for binding to 50K. When
the plates were coated with the H/120 fragment, \( \alpha 4 \beta 1 \) but not \( \alpha 5 \beta 1 \)
competed with MIA for binding to H/120 (Fig. 9D).

Next, we wanted to analyze whether the regulation of MAPK activity by
MIA is modulated by direct binding of MIA to integrins. The 50K fragment
was used in the experiments because of its inhibitory effect on MIA inte-
grin interaction. Western blots with antibodies against phosphorylated ERK1/2
to specifically analyze ERK activity after MIA and MIA plus 50K treatment
were performed (Fig. 10A). MIA treatment showed inhibition of ERK activity,
which was reversed dose-dependently by the 50K fragment. Addition-
ally, \( \beta 1 \)-integrin deficient cells (GD25-\( \beta 1 \)) were used in comparison with
wild type cells to prove the regulation of MAPK activity by MIA via inte-
grins. No change in ERK activity was observed after treating the \( \beta 1 \) integrin-
negative cells with MIA, whereas the wild type cells showed clear reduction
as expected (Fig. 10B).

DISCUSSION

In this study we aimed to analyze signaling pathways and putative
receptor molecules of MIA. Previous studies revealed direct interaction

\[ \text{figure legend:} \]

\[ \text{fibrinectin type I domain} \]
\[ \text{fibrinectin type II domain} \]
\[ \text{fibrinectin type III domain} \]
\[ \text{connecting segment} \]
\[ \text{synergy site} \]
\[ \text{RGD} \]
\[ \text{arginine, glycine, aspartic acid} \]
\[ \text{central cell binding domain} \]
\[ \text{heparin binding domain} \]

\[ \text{FIGURE 8. Schematic diagram of fibronectin. A, schematic diagram of one fibronectin monomer and its binding properties. B, 50-kDa gelatin binding domain (50K). C, 120-kDa central cell binding domain (H/120).} \]
of MIA with several matrix proteins such as fibronectin or laminin (19); however, cell surface receptors for MIA or MIA-regulated signaling pathways have not been characterized until now.

Analyzing the MAP kinase pathway, we found strong reduction of ERK1/2 but not of p38 and c-Jun N-terminal kinase activity after MIA treatment. To investigate whether this effect on ERK1/2 activity was a consequence of MIA binding to fibronectin inducing cellular detachment, we examined the effect of RGD peptides, simulating cellular detachment from fibronectin, on ERK1/2 phosphorylation. Incubation of melanoma cells with RGD peptides induced only a weak reduction of ERK1/2 phosphorylation. These results implied that MIA influences ERK1/2 activity either via a different binding motive on the integrins or by as yet unknown cell surface receptors.

FACS analysis revealed that MIA binds directly to the cell surface. Subsequent experiments with fibronectin positive and negative mouse embryonic fibroblasts showed no differences in MIA binding to the cell surface. Therefore, we hypothesized the existence of a cell surface molecule directly binding MIA and excluded the possibility of MIA binding to cells via matrix proteins bound to the cell surface.

To characterize MIA binding partners, we performed co-immunoprecipitation analysis with consecutive SDS-PAGE and silver staining and, additionally, far Western blots. Molecules of ~70, 80, and 100 kDa
were found to bind to MIA in both experiments. Because several publications showed this pattern after staining for integrins α4 and α5 (16–18), we analyzed whether these proteins were detectable in our co-immunoprecipitation experiments. Therefore, specific antibodies against integrin α4 and integrin α5, respectively, were used to analyze the MIA co-immunoprecipitate by Western blot. Both antibodies revealed specific staining at 70 and 80 kDa, whereas antibodies against integrins α2 and α3 did not show staining. It is known that the 150-kDa pro-protein forms of α4 and α5 are proteolytically cleaved by proprotein convertases. α4 is cleaved in the post-endoplasmic reticulum into a C-terminal 70-kDa fragment and a N-terminal 80-kDa fragment that are noncovalently linked (21, 22). For both α4 and α5, it has been shown that only the processed form is present on the membrane surface of cells (16, 22, 23). The antibodies used in our experiment were directed against the C termini of α4 and α5 integrins (16, 18, 24). In RIPA lysates of human melanocytes and Mel1m cells, the processed C-terminal fragment of α4 integrin was detected (Fig. 4C). This processed form was bound by MIA together with a 80-kDa form as seen by silver staining of the co-immunoprecipitates and in the far Western blot. For α5, however, we mainly found the pro-protein of the fragment in melanocyte RIPA lysates (130–140 kDa). In Mel1m lysates lower bands at ~70–80 kDa were detected, which presumably reflect the processed forms (see arrows in Fig. 4C). The staining further revealed that only the large form of α5 integrins is present in normal cells, whereas in melanoma cell lines both the unprocessed α5 and the processed forms, are detectable.

To verify the results of MIA binding to integrins α4 and α5, ELISA experiments were performed suggesting that MIA is able to directly bind to α4β1 and α5β1, respectively. These data were further supported by FACS analysis using the antibody 12G10 specifically detecting activated integrins. Our data indicate that MIA not only binds to integrins but also down-regulates their activity. Considering the fact that integrins are involved in ERK1/2 signaling (19), these results confirmed our data that ERK1/2 phosphorylation decreased in melanoma cells after incubation with MIA.

To further specify MIA binding to α4β1 and α5β1 we performed competition experiments with the fibronectin fragments 50K and H/120 (a schematic model is depicted in Fig. 8). It is known that 50K contains an RGD site and binds specifically to α5β1; H/120 binds specifically to α4β1 via the connecting segment (IIICS) (14). We found that the 50K fibronectin fragment competed with MIA binding to α5β1 dose-dependently. It also competed with MIA binding to α4β1, although to a lesser extent. This might be due to the fact that MIA is able to directly bind to the 50K fragment at two different sites (FN6 and the RGD site) (10), possibly resulting in sequestration of MIA by the 50K fragment and inhibition of binding to the integrin. Additionally, this hinted to the possibility that the 50K fragment either binds to the same site of the integrins MIA bind to or it covers the MIA-integrin binding epitope. Otherwise, MIA bound by the 50K fragment would also be able to bind to the integrins.

Interestingly, when performing the experiments with low concentration of the FN fragment H/120, it did not compete with MIA on α4β1 and α5β1. At higher concentrations it even increased MIA binding mainly to α5β1 integrin. It was shown in previous studies that MIA is able to bind to domains of the H/120 fragment (FN14 and the connecting segment IIICS) (10). Potentially, a complex was formed in which the H/120 fragment (bound by additional MIA molecules) was bound to the integrin α5β1 via MIA. If H/120 binds to MIA without covering the MIA integrin-binding site, MIA would be able to support indirect binding of H/120 to α4β1 integrins. We therefore speculated that the 50K fibronectin fragment blocked the MIA integrin-binding site, whereas the H/120 fibronectin fragment did not interfere with MIA integrin binding. However, plating the fibronectin fragments first and, as a second step, incubating with MIA and subsequently competing with soluble integrins showed clear competition of MIA with soluble α5β1 on 50K and α4β1 on H/120. Only a slight reduction of MIA binding was found competing binding to 50K with α4β1 or binding to H/120 with α5β1. These results are explained by the fact that α4β1 and 50K and α4β1 and H/120, respectively, do not interact. However, a minor amount of MIA might be sequestered by α4β1 or α5β1. Consecutive assays revealed that the regulation of ERK activity by MIA is via direct binding of MIA to integrins. Integrins α4β1 and α5β1 have been shown to play an important role in melanoma development and progression (for review see Ref. 28).

Expression of both has been proven to be elevated in primary and metastatic melanoma (25–27). Furthermore, expression of the α4 subunit is associated with melanoma cell accumulation of disseminated cells in distant tissues (27). α4β1 is usually expressed as a first step of extravasation to support binding to VCAM-1 expressed on activated endothelial cells. Binding of integrins to matrix proteins is a tightly regulated process. This is best exemplified in migrating cells where cells...
form a zone of attachment in the front and detachment at the rear. We hypothesize that the control of integrin activity by MIA regulates migration of melanoma cells.

In summary, this study revealed that MIA directly interacts with integrin α4β1 and α5β1. This interaction modulates integrin activity and additionally leads to down-regulation of integrin signaling via ERK1/2.

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