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The Golgi-Associated Protein p115 Mediates the Secretion of Macrophage Migration Inhibitory Factor

Melanie Merk,*‡ John Baugh,‡ Swen Zierow,*† Lin Leng,* Utpal Pal,* Seung Joon Lee,‡ Antje D. Ebert,§ Yuka Mizue,* John O. Trent,¶ Robert Mitchell,¶ Walter Nickel,§ Paula B. Kavathas,* Jürgen Bernhagen,† and Richard Bucala3*

Macrophage migration inhibitory factor (MIF) is a leaderless protein that is secreted from cells by a specialized, nonclassical export pathway. The release of MIF nevertheless is regulated and its production in response to different inflammatory, mitogenic, and hormonal stimuli plays an important role in diverse physiologic and pathologic processes. We report herein the identification of the Golgi complex-associated protein p115 as an intracellular binding partner for MIF. MIF interacts with p115 in the cytoplasm and the stimulated secretion of MIF results in the accumulation of both proteins in supernatants, which is consistent with MIF release from cells in conjunction with p115. The depletion of p115 from monocytes/macrophages decreases the release of MIF but not other cytokines following inflammatory stimulation or intracellular bacterial infection. Notably, the small molecule MIF inhibitor 4-iodo-6-phenylpyrimidine inhibits MIF secretion by targeting the interaction between MIF and p115. These data reveal p115 to be a critical intermediary component in the regulated secretion of MIF from monocytes/macrophages. The Journal of Immunology, 2009, 182: 6896–6906.

In contrast to the “classical” pathway for secretion, in which a hydrophobic leader sequence targets proteins for export via the endoplasmic reticulum and Golgi (6–8), nonclassical protein secretion is less well understood (9–11). Several distinct mechanisms have been revealed for particular proteins such as vesicular transport via lysosomes or exosomes, plasma membrane shedding, and direct translocation across the membrane by specialized transporters. Potential secretory pathways that have been described for IL-1β, for instance, include cell surface blebbing and the formation of microvesicles that lyse in the extracellular space (12) and the fusion of endolysosomal vesicles with the cell surface plasma membrane (13). Fibroblast growth factor (FGF) 2, which is another unconventionally secreted cytokine, appears to cross the membrane in an ATP- and membrane-potential independent manner (10, 14, 15) and requires the action of phosphatidylinositol phosphate kinases (PIP-K) (16, 17). There is evidence that MIF may be associated with vesicles in particular cell types (18, 19), and a recent report has shown that MIF secretion is reduced by glyburide (5). Glyburide inhibits ATP-binding cassette transporters; it targets numerous proteins such as ion channels, enzymes, and other transporters (20, 21).

To gain insight into the protein components and the mechanisms responsible for MIF release, we performed a yeast two-hybrid interaction study to identify intracellular proteins that might bind MIF and mediate its export. We report herein the identification of the Golgi-associated protein p115 (22) as a binding partner for MIF, and we provide evidence for the p115-dependent release of MIF from human monocytes/macrophages. We further show that a recently described, small molecule MIF inhibitor, 4-iodo-6-phenylpyrimidine (4-IPP) (23), targets MIF secretion by influencing the interaction between MIF and p115.

Materials and Methods

Yeast two-hybrid studies

A human pituitary cDNA library and the CytoTrap yeast two-hybrid system were purchased from Stratagene and used according to the manufacturer’s instructions. Briefly, cdc25H yeast cells were cotransformed with a MIF-bait plasmid (pSOS-MIF) and a library of human pituitary cDNA...
Aldrich) and PMA-differentiated THP-1 macrophages were stimulated

p115702–962. Values are plotted as percent absorbance at 405 nm relative to
two mAb clones MAB289 (5) and 3H2F (25) were used as capture Abs.

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none imine (NAPQI) (24) and 4-iodo-6-phenylpyrimidine (4-IPP) was
from Roche. The chemical modification of MIF by
scribed previously (4) and biotinylated using a Biotin Protein Labeling Kit

LinkTM-sulfo-NHS-LC biotin (Pierce). Human MIF was prepared as de-
p115 capture assay, recombinant p115702–962 was biotinylated using EZ-
cosis of interactions was tested by retransformation of cdc25H cells with

(28).

observed by fluorescent microscopy. GM130 served as marker for the

golgi (28).

interaction between MIF and p115 was visualized using an anti-V5 Ab to detect the V5 tag on the p115 C terminus. For endogenous common immunoprecipitation, LNCap cell lysates were prepared us-
ining radioimmunoprecipitation. Buffer B was used to solubilize endogenous

p115 and MIF (27). Endogenous MIF was immunoprecipitated and protein

complexes were resolved by SDS-PAGE and visualized with anti-p115 Ab

(CB1009; Calbiochem).

Confocal immunofluorescence

PBMCs (2.5 × 10^9/ml) were cultured and differentiated on glass slides (BD

Falcon) washed in PBS, and fixed and permeabilized using 3.7% formal-
dehyde and 0.1% Triton-X-100 in PBS (50 min at 37°C). After blocking in

5% goat serum (Sigma-Aldrich) for 20 min, the slides were incubated

overnight at 4°C, followed by washing with TBST and blocking with

Superblock (Pierce). Biotinylated MIF (150 nM) was added in duplicate to

wells containing biotinylated MIF or p115702–962 alone. Values are plotted as percent absorbance at 405 nm relative to

p115702–962 was added along with increasing concentrations of unlabeled

p115702–962. The cells were resuspended in RPMI 1640 medium supplemented with

β-actin were designed and synthesized by SuperArray. The quantitative

PCR profile was done according to SuperArray’s manual and specific prod-

uct amplification was verified with a melting curve.

Coimmunoprecipitation and Western blotting

PbSOS-MIF, PbSOS-Coll, or PbSOS-MAFB. Library plasmids that specific-

ically interacted with MIF were identified by DNA sequencing.

p115 cloning and recombinant protein preparation

A truncated p115 protein (p115702–962) representing the clone identified in

the yeast two-hybrid screen was subcloned into the pET22b vector, which

contained a carboxy-terminal histidine tag (Novagen) and was expressed in

Escherichia coli BL21(DE3). The crude bacterial extract was purified us-

ing a HiTrap chelating HP column (Amersham Biosciences). Protein purity was

verified by SDS-PAGE/Coomasie staining and Western blotting. For the

p115 capture assay, recombinant p115702–962 was biotinylated using EZ-

to 4-µm excess of compound at room temperature overnight (23). The modified MIF was dialyzed against

PBS. For expression in mammalian cells, full-length p115 was cloned into

cDNA3.1 (Invitrogen).

MIF/p115-binding studies

Ninety-six well Nunc Immuno-Module plates were coated with 1 µM

p115702–962/well. The plates were washed with TBST and blocked with

Superblock (Pierce). Biotinylated MIF (150 nM) was added in duplicate to

wells along with increasing concentrations of unlabeled MIF or lysozyme

as control. Incubation was continued overnight at 4°C, followed by wash-

ing with TBST. The bound, biotinylated MIF was detected by adding

streptavidin-conjugated alkaline phosphatase (R&D Systems) for 1 h at

room temperature, followed by washing and detection of the alkaline phos-

phatase with p-nitrophenyl phosphate (Sigma-Aldrich). For a reverse bind-

ing assay, 1 µM MIF was coated on wells and 150 nM biotinylated

p115702–962 was added along with increasing concentrations of unlabeled

p115702–962. Values are plotted as percent absorbance at 405 nm relative to

wells containing biotinylated MIF or p115702–962 alone.

For measurement of MIF’s immunoreactivity in an ELISA system, the

two mAb clones MAB289 (5) and 3H2F (25) were used as capture Abs.

Cell culture studies

Cell lines were obtained from American Type Culture Collection, with the

exception of the HEK293 cells, which were purchased from Invitrogen. For
differentiation of THP-1 monocytos into macrophages, 50 ng/ml PMA

(Sigma-Aldrich) was added for 24 h, after which the medium was changed

and the cells were cultured for an additional 48 h (26).

The MIF secretion assay was performed as described previously (5).

Briefly, cells first were synchronized for 3 h in medium containing 1%

FCS. THP-1 monocytos or primary human PBMC-derived macrophages

(1 × 10^6/ml) were stimulated with 10 µg/ml LPS 0111:B4 (Sigma-

Aldrich) and PMA-differentiated THP-1 macrophages were stimulated with

0.1 µg/ml LPS. For studies of MIF inhibitors, the test compounds

were added 1 h before LPS stimulation. Cytokine levels in the supernatants

were analyzed by ELISA (eBioscience), with the exception of the MIF

ELISA, which was performed as described previously (25). Unless stated

otherwise, background immunoreactivity was subtracted from the stimu-

lated cytokine level. Cell viability was verified using an lactate dehydro-

genase (LDH) assay (Roche).

RNA isolation and quantitative PCR

RNA was isolated using RNAeasy from Qiaqen. Reverse transcription was performed using StrataScript according to the manufacturer’s instructions (Stratagene). For quantitative PCR, the following primers were used: MIF forward, 5'-CGGACAGGGTCTACATCAA-3' and MIF reverse, 5'-CT

TAGGCCGAAA-GTGGTATTGTT-3' and β-actin forward, 5'-CGATGCA

GAAGGAGATCA-CTG-3' and β-actin reverse, 5'-CGATCACCAGC

GAGTACT TG-3'. The amplification profile included denaturation at 95°C for

5 min followed by annealing and elongation for 1 min at 60°C for a total of

40 cycles. For the p115 quantitative PCR, primers for p115 and

β-actin were designed and synthesized by SuperArray. The quantitative

PCR profile was done according to SuperArray’s manual and specific prod-

uct amplification was verified with a melting curve.

Primary cell cultures

Human PBMCs were isolated by Ficoll-Hypaque gradient centrifugation.
The cells were resuspended in RPMI 1640 medium supplemented with
20% human AB serum (Cambrex) and plated at 2.5 × 10^6 cells/well in 4-

in 24-well tissue culture plates. After 2 h of culture, the adherent cells were

washed extensively with PBS and cultured for 1 wk to allow differentiation

into monocyte-derived macrophages. THP-1 cells (1 × 10^6/ml) were in-
fected with Chlamydia trachomatis serovar L1 at a MOI of 10 for 1 h at

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Identification of p115 as a new binding partner of MIF. A, Tertiary screen confirming the specific binding of 15 cDNA clones with MIF. Yeast were cotransformed with the 20 putative binding partners identified in the secondary screen plus either MIF or control plasmids. Top left plate shows the positive and negative controls. Remaining plates show galactose-dependent growth of 15 MIF-specific interacting clones (plated in triplicates). B, Schematic structure of p115. Top, Full-length p115 with its globular head (hatched), four coiled-coiled domains (black), and an acidic tail (gray). Bottom, Carboxyl terminus of p115 (residues 702–962) that interacts with MIF. C, Purified recombinant p115702–962 revealed by SDS-PAGE followed by Coomassie staining (left panel) and Western blot (right panel). D, MIF binding to p115 demonstrated by a competitive binding assay. Left panel, MIF binds to immobilized p115702–962 in a concentration-dependent manner. Right panel, p115702–962 binds to immobilized MIF in a concentration-dependent manner. Lysozyme served as a negative control protein. Results are expressed as mean ± SD of duplicate measurements and are representative of three independent experiments. E, Coimmunoprecipitation of MIF and p115. Upper panel, V5 epitope-tagged p115 was expressed in COS-7 cells and the cells lysed 48 h later. p115 containing protein complexes were coimmunoprecipitated with the addition of recombinant MIF and anti-MIF Ab or after the addition of GST-MIF; p115 was visualized by Western blot using an anti-V5 Ab. Control IgG1 and GST did not coprecipitate p115. IB, Immunoblot. Lower panel, Endogenous MIF-p115 complexes were coimmunoprecipitated from LNCap prostate carcinoma cells. MIF was immunoprecipitated with a monoclonal anti-MIF Ab; p115 was detected by Western blot using a polyclonal anti-p115 Ab.

**Results**

Identification of p115 as an intracellular MIF-binding protein

To identify intracellular proteins that mediate MIF secretion, we used the CytoTrap yeast two-hybrid screen, which relies on the cytoplasmic rescue of a temperature-sensitive mutation in the Ras signaling pathway (33). MIF cDNA served as “bait” and a human pituitary cDNA library was the “prey” (34). After cotransformation of bait and prey plasmids into the Saccharomyces cerevisiae strain cdc25H, 90 colonies were identified in a primary screen. The secondary and tertiary screenings ruled out 75 colonies as false positives that showed growth in the absence of the library protein. Prey proteins in the remaining 15 colonies bound specifically to MIF (Fig. 1A). By DNA sequencing, one of the positive clones contained the carboxyl-terminal 780 bases of the cDNA for MIF (Fig. 1A). By DNA sequencing, one of the positive clones contained the carboxyl-terminal 780 bases of the cDNA for MIF (Fig. 1A). By DNA sequencing, one of the positive clones contained the carboxyl-terminal 780 bases of the cDNA for MIF (Fig. 1A). By DNA sequencing, one of the positive clones contained the carboxyl-terminal 780 bases of the cDNA for p115. The remaining 14 colonies all contained the coding sequence of MIF; these may be regarded as an internal positive control because MIF forms a homotrimer (35, 36). p115 was characterized initially as a vesicle-docking protein that is localized predominantly to the cytosolic side of vesicular tubular intermediate clusters and the cis-Golgi (22, 37, 38). The carboxyl-terminal region of p115 consists of four coiled-coiled domains and a short acidic tail (37) (Fig. 1B).

To biochemically confirm the interaction between MIF and p115, we produced the carboxyl-terminal region of p115 (p115702–962) in E. coli (Fig. 1C) and evaluated its binding to MIF in an in vitro competition binding assay. As shown in Fig. 1D, increasing concentrations of MIF, but not the equimolar addition of a control protein (lysozyme), inhibited the interaction of biotinylated MIF with p115. Protein-protein interaction was verified by performing the reverse assay, i.e., measuring the binding of biotinylated p115702–962 to immobilized MIF.

The intracellular interaction between MIF and p115 was further investigated by coimmunoprecipitation. COS-7 cells that were transfected with a V5 epitope-tagged p115 served as a source for...
After the addition of recombinant MIF, a MIF-p115 complex was coprecipitated using an anti-MIF Ab. p115 also coprecipitated after adding GST-tagged MIF (Fig. 1E). An endogenous interaction between p115 and MIF also was observed by Western blot analysis for p115 after immunoprecipitation with anti-MIF in the prostate carcinoma cell line LNCap (27). These results, taken together, confirm an interaction between p115 and MIF in mammalian cells.

Macrophage activation leads to a redistribution of cellular MIF
We studied the cellular distribution of MIF in macrophages in response to LPS stimulation. In unstimulated macrophages, MIF appears present throughout the cytoplasm and p115 is predominantly in the perinuclear area in a pattern that is consistent with the Golgi (Fig. 2A), which is in agreement with previous reports (22, 38). In the time course study of LPS-activated primary macrophages, MIF gradually redistributes from the cytoplasm to a plasma membrane-proximal area and a portion of p115 now also appears dispersed in the cytoplasm. Furthermore, in the course of LPS stimulation, a colocalization of MIF and p115 at the area of the Golgi and in the cytoplasm can be observed (Fig. 2B).

MIF release from human monocytes
MIF is released from cells in both a regulated and a specific manner (5, 39). We stimulated human THP-1 monocytes with LPS and observed an increase in the concentration of MIF in supernatants that was detectable at 2 h and reached a plateau at 4 h (Fig. 3A). The observed increase in the supernatant content of MIF was not the result of cell death as assessed by LDH analysis, and it was not accompanied by an increase in the level of MIF or p115 mRNA measured over 6 h (Fig. 3B and data not shown). These data indicate that the initial release of MIF from THP-1 monocytes occurs from preformed pools and does not require the transcription of MIF mRNA. The results are in accord with previous reports that were based on the immunostaining and in situ hybridization of murine tissues after LPS administration in vivo (2, 3).

FIGURE 2. Immunostaining of p115 and MIF in macrophages. A, Freshly isolated PBMCs were plated on a four-chamber culture slide (2.5 × 10⁶ cells/chamber). The cells were cultivated for 7 days to allow differentiation, fixed with formaldehyde, and permeabilized with Triton X-100. MIF (red) and p115 (green) were visualized by immunostaining with specific Abs (1/200). The nucleus was stained using To-Pro3 (blue). B, Macrophages that were stimulated with LPS (10 μg/ml) for 0, 0.5, 1, and 4 h were fixed with formaldehyde and permeabilized with Triton X-100. MIF and p115 were visualized by immunostaining with specific Abs. To-Pro3 was used as a nuclear dye. Images are representative for n = 60 cells. Scale bar, 10 μm.

FIGURE 3. Cell stimulation increases MIF secretion without affecting MIF or p115 mRNA levels. A, Time course of MIF secretion from LPS-stimulated, THP-1 monocytes. THP-1 cells (1 × 10⁶/ml) were treated with LPS (10 μg/ml) or PBS (control) and supernatants were collected at the indicated times for measurement of MIF by specific ELISA. Results are expressed as mean ± SE of duplicate measurements from two independent experiments (n = 4). Values of p were calculated by Student’s t test for all time points. *, p < 0.01 for stimulated vs unstimulated cells. B, MIF and p115 mRNA levels in LPS-stimulated THP-1 monocytes. RNA was isolated at the indicated time points and analyzed by quantitative PCR. Results are expressed as mean ± SE of three independent experiments.

p115 is necessary for the release of MIF from THP-1 monocytes
p115 plays a role in vesicle transfer from the endoplasmic reticulum to the Golgi complex (40). MIF does not enter the Golgi and it was of interest to examine whether p115 is necessary for the release of MIF in response to activating stimuli. We generated RNAi constructs for the gene-specific silencing of p115 and tested
We next examined whether p115 knockdown reduced the amount of MIF produced by LPS-stimulated THP-1 monocytes. Transfection of the p115-1 or p115-2 RNAi plasmids resulted in a 40–60% decrease in MIF release in response to LPS (Fig. 4A). Of note, the LPS-stimulated secretion of two conventionally secreted cytokines, TNF-α and IL-6, which have hydrophobic leader sequences, was not affected by a reduction in cellular p115 protein. These data point to the potential specificity of p115 in mediating MIF secretion. TNF-α and IL-6 secretion also affirms that the depletion of p115 by the RNAi approach used herein does not appreciably affect the functioning of the ER/Golgi pathway. We further studied the effect of depleting GM130, which is a p115 binding partner that interacts with the carboxyl-terminal acidic tail of p115 (22), but there was no measurable effect of GM130 depletion on MIF secretion (data not shown).

To gain a broader understanding of the role of p115 in unconventional protein secretion, we studied the export of the two structurally related cytokines FGF-2 and FGF-4. Although both proteins are released from cells in response to tissue injury (41–43), FGF-4 is secreted conventionally, whereas FGF-2 translocates directly across the plasma membrane (15). Using a previously established doxycycline-inducible expression system (32), we measured both total and cell surface-associated FGF-2 or FGF-4 protein (Fig. 4B). Cellular depletion of p115 did not influence the export of either FGF-2 or FGF-4, while the secretion of FGF-2 was inhibited by depletion of PIP-K as previously reported (16). The finding that the release of both conventionally secreted cytokines (TNF-α, IL-6, FGF-4) and the unconventionally secreted cytokine FGF-2 is not affected by p115 depletion suggests a specific role for p115 in the stimulated export of MIF.

**FIGURE 4.** p115 depletion inhibits MIF secretion. **A. Upper panel.** THP-1 cells were transfected with RNAi constructs specific for p115 (p115-1, p115-2) or a control plasmid (mock) and analyzed for cellular p115 content by Western blotting. **Lower panel,** THP-1 monocytes (1 X 10⁶/ml) were transfected with p115 RNAi or mock RNAi plasmid and stimulated with LPS for 4 h. Supernatants were collected and analyzed for MIF, TNF-α, and IL-6 by specific ELISA. Results are expressed as the mean ± SE of four independent experiments and data are cytokine levels after subtraction of baseline. Statistical significance was determined for each p115 RNAi construct against mock siRNA by unpaired Student’s t test; †, p < 0.05 and ‡, p < 0.01. **B. Upper panel,** HeLa cells were transfected with mock, p115-2, or PIP-K RNAi constructs and analyzed for cellular p115 content by Western blotting. **Lower panel,** FGF-GFP fusion protein production was induced by doxycycline 96 h after transfection of cells with mock, p115-2, or PIP-K RNAi. FGF-GFP fusion proteins were measured after an additional 24 h as total or cell surface fluorescence. Data are shown relative to the background level of GFP.

We used an adenovirus construct to deliver p115 siRNA to differenti- ated THP-1 macrophages and observed that adenoviral transfection reduced significantly intracellular p115 protein levels (Fig. 5C). The transfection of adenovirus encoding p115 siRNA, but not a control adenovirus, significantly reduced LPS-stimulated MIF release over the 24 h experimental period as demonstrated by Western blot analysis (Fig. 5D) and ELISA (Fig. 5E). Of note, the decrease in MIF secretion in p115-depleted cells is not due to cell death or permeabilization as assessed by LDH assay. LDH levels were measured over the course of the experiment; LDH concentration never exceeded 14 mU/ml (<5% total cellular LDH), and no statistical differences between mock-treated and p115-depleted human THP-1 monocytes acquire many of the characteristics of mature, primary macrophages upon differentiation with PMA, such as an increase in the expression of the LPS coreceptor CD14 (26). Differentiated THP-1 macrophages thus respond to low doses of LPS and show a plateau in the MIF secretion response at a LPS concentration of 0.1 μg/ml (Fig. 5A). Differentiated THP-1 macrophages also secrete ~3-fold more MIF protein than undifferenti- ated THP-1 monocytes. Of note, ELISA and Western blot analyses of LPS-stimulated THP-1 macrophages showed a coordinated increase in secreted MIF and p115 and a corresponding decrease in the cellular protein levels over the observed time period of 24 h (Fig. 5B). Moreover, the supernatants of unstimulated macrophages contained only a modest amount of MIF and p115, and no apparent reduction in the cellular MIF and p115 content was measurable. These results taken together suggest that MIF and p115 are cosecreted. Further evidence that MIF and p115 are cosecreted comes from the observation that glyburide, an pharmacologic inhibitor known to inhibit the secretion of MIF (5), also shows an inhibitory effect on the secretion of p115 (data not shown). Of note, glyburide is also known to inhibit the release of other unconventionally secreted proteins (44, 45).

We used an adenovirus construct to deliver p115 siRNA to differenti- ated THP-1 macrophages and observed that adenoviral transfection reduced significantly intracellular p115 protein levels (Fig. 5C). The transfection of adenovirus encoding p115 siRNA, but not a control adenovirus, significantly reduced MIF release over the 24 h experimental period as demonstrated by Western blot analysis (Fig. 5D) and ELISA (Fig. 5E). Of note, the decrease in MIF secretion in p115-depleted cells is not due to cell death or permeabilization as assessed by LDH assay. LDH levels were measured over the course of the experiment; LDH concentration never exceeded 14 mU/ml (<5% total cellular LDH), and no statistical differences between mock-treated and p115-depleted cells with mock, p115-2, or PIP-K RNAi. FGF-GFP fusion proteins were measured both total and cell surface-associated FGF-2 or FGF-4 protein (Fig. 4B). Cellular depletion of p115 did not influence the export of either FGF-2 or FGF-4, while the secretion of FGF-2 was inhibited by depletion of PIP-K as previously reported (16). The finding that the release of both conventionally secreted cytokines (TNF-α, IL-6, FGF-4) and the unconventionally secreted cytokine FGF-2 is not affected by p115 depletion suggests a specific role for p115 in the stimulated export of MIF.
FIGURE 5. MIF and p115 are secreted from differentiated THP-1 macrophages. A, Dose-dependent secretion of MIF from LPS-stimulated THP-1 macrophages. Cells (1 × 10^6/ml) were stimulated with the indicated dose of LPS for 4 h and the supernatants were assayed for MIF content by ELISA. Results are expressed as mean ± SD of duplicate experiments, each measured in duplicate (n = 4). B, Left panel, Differentiated THP-1 macrophages were stimulated with 0.1 μg/ml LPS or PBS (control) and the supernatants were collected at the indicated time points. MIF concentration was measured by ELISA; p115 release was determined by immunoblotting and subsequent densitometric analysis. Right panel, Cell lysates of THP-1 macrophages stimulated with LPS or control (PBS) were prepared at the indicated time points. Intracellular MIF concentration was measured by ELISA and intracellular p115 level was determined by Western blot and densitometric analysis. The ELISA results are expressed as mean values ± SD of two independent experiments measured in duplicate. Densitometric results are expressed as mean values ± SD of at least two independent experiments. Statistical significance was determined by Student’s t test comparing LPS-stimulated vs control time point; *, p < 0.05 and **, p < 0.01. C, Differentiated THP-1 macrophages were transfected with a MOI of 50 adenovirus-encoding siRNA against p115 or a mock control siRNA. Ninety-six hours after transfection, the cells were lysed and the protein content was analyzed by Western blot and densitometry. The results are expressed as mean values ± SE of three independent experiments and statistical significance was determined by Student’s t test; *, p < 0.001. D, LPS-stimulated secretion of MIF in p115-depleted or mock-treated THP-1 macrophages. Supernatants were collected at the indicated time points and analyzed for export of MIF and p115 by Western blot. E, Time course of LPS-stimulated MIF secretion in control or p115 siRNA-treated THP-1 macrophages. Supernatants were collected at the indicated time points and MIF content was assayed by ELISA. Supernatants also were assayed for TNF-α, IL-6, and IL-1β by ELISA. The results are expressed as mean values ± SE.
FIGURE 6. MIF and p115 are secreted from primary macrophages. A, Adenovirus-mediated depletion of p115 reduces p115 protein levels in primary human macrophages. Human peripheral blood monocyte-derived macrophages (1 × 10⁶/ml) were transfected with 100 MOI of adenovirus-carrying siRNA against p115 or a mock control. The cells then were analyzed 48 h later for p115 content by Western blot. Knockdown of p115 was evaluated by densitometry. The results are expressed as mean values ± SD of three independent experiments and statistical significance was determined by Student’s t test; *p < 0.001. B, LPS-stimulated MIF secretion is reduced in p115 siRNA, but not mock siRNA-treated primary macrophages. MIF secretion was stimulated with 10 ng/ml LPS for 4 h and the supernatants were analyzed for MIF content by ELISA. The results are expressed as mean values ± SD of three independent experiments, each measured in duplicates (n = 4). Data are MIF levels after subtraction of baseline. Statistical significance was determined by an unpaired Student’s t test; *p < 0.001. C, Bone marrow-derived primary macrophages (0.5 × 10⁶/ml) were stimulated with 1 μg/ml LPS or control (PBS) and the supernatants were collected at the indicated time points. MIF concentration was measured by ELISA and p115 content was determined by immunoblotting. Results are representative of three independent experiments. Statistical significance was determined by Student’s t test comparing LPS-stimulated vs control time point; *p < 0.01.

FIGURE 7. p115 knockdown inhibits MIF secretion in C. trachomatis-infected human monocytes. A, THP-1 monocytes (1 × 10⁶/ml) were infected with 10 MOI of C. trachomatis. Supernatants were collected at the indicated time points and analyzed for MIF release by ELISA. B, Differentiated THP-1 macrophages were treated with 50 MOI of adenovirus encoding a p115 or a control (mock) siRNA 96 h before infection with C. trachomatis. Supernatants were collected at the indicated time points and the levels of MIF and TNF-α were analyzed by ELISA. Results are expressed as mean ± SE of one experiment measured in triplicates and the data are total cytokine levels. Results are representative of three independent experiments. Statistical significance was analyzed by an unpaired Student’s t test; *p < 0.01.
The effect of *C. trachomatis* infection on MIF production in differentiated THP-1 macrophages that had been pretreated with adenovirus encoding either a mock control or a p115 siRNA. p115 depletion had no effect on the infection or replication rate of organisms in macrophages, as measured by flow cytometry for the *C. trachomatis* Ag LPS (24 h after infection: mock siRNA: 38 ± 5% vs...
p115 siRNA: 39 ± 8%, p = NS; data not shown). Nevertheless, macrophages treated with p115 siRNA showed a significant decrease in MIF secretion after *C. trachomatis* infection. By contrast, the secretion of TNF-α, which was evident at 8 h after infection, was unaffected by p115 siRNA (Fig. 7B). These data indicate that cellular p115 plays an important role in mediating the secretion of MIF by macrophages in response both to a defined microbial ligand such as LPS, as well as to live infection by an intracellular pathogen such as *C. trachomatis*.

The small molecule inhibitor 4-IPP MIF secretion by targeting the MIF-p115 interaction

Prototypic small molecules have been identified that bind to the MIF amino-terminal, tautomerase region (24, 49). Mitchell and colleagues recently reported on a suicide substrate, 4-IPP, which was identified by a computational virtual screening for MIF antagonists, and cocrystallization of MIF and 4-IPP demonstrated that it covalently modifies the MIF amino terminus and inhibits MIF-dependent cell motility and growth in vitro (23). It was of interest to investigate whether 4-IPP, which is cell permeable, inhibits the stimulated release of MIF. Because of previous studies showing that the covalent modification of MIF by a small molecule may affect its immunorecognition in a two-Ab, sandwich ELISA (24), we first determined whether 4-IPP influences the detection of MIF by ELISA. Indeed, MIF that had been incubated with 4-IPP showed increased immunorecognition by a MIF-specific mAb (clone MAB289) when compared with native MIF (Fig. 8A). By contrast, when analyzing 4-IPP-modified MIF with a different anti-MIF mAb (clone 3H2F), a diminished recognition compared with native MIF was observed (>2-fold; data not shown). We concluded that 4-IPP significantly alters the native conformation of the MIF protein, and we therefore used Western blotting for analyzing the effect of 4-IPP on MIF secretion. 4-IPP modification did not affect MIF detection by Western blot (data not shown).

We found that increasing concentrations of 4-IPP dose-dependently block the release of MIF, with 1 μM 4-IPP (10-fold molar excess) resulting in ~80% inhibition of MIF secretion (Fig. 8B). 4-IPP also inhibits the basal secretion of MIF (Fig. 8C). Of note, 4-IPP effectively inhibits the release of both MIF and p115 from LPS-stimulated THP-1 macrophages and, correspondingly, the cytoplasmic levels of MIF and p115 did not decrease when LPS-stimulated cells were treated with 4-IPP (Fig. 8D). 4-IPP did not affect the release of TNF-α and IL-1β (Fig. 8E), while reducing the secretion of MIF from monocytes infected with *C. trachomatis* (Fig. 8F). We furthermore studied the influence of the reversible MIF-binding molecule (S,R)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester (ISO-1) (49) on MIF secretion from stimulated THP-1 macrophages but observed no effect (data not shown). The observation that 4-IPP enhanced the recognition of MIF by an anti-MIF mAb (Fig. 8A) suggests that 4-IPP imparts a significant conformational affect on MIF that may also influence its binding to p115. We tested this possibility by examining the effect of 4-IPP on the competitive binding of MIF to p115 in vitro. As shown in Fig. 8G, 4-IPP, but not the small molecules ISO-1 or NAPQI (24, 49), increased MIF binding to p115. The enhanced binding of MIF to p115 in the presence of 4-IPP thus may play a role in the inhibitory action of 4-IPP on MIF release from cells, perhaps by interfering with additional protein interactions necessary for secretion.

Discussion

MIF plays an important upstream role in the regulation of diverse cellular responses (39, 50–52) and its role in human pathology has been emphasized by the finding that high expression MIF alleles are associated with the incidence or the severity of inflammatory and oncologic diseases (25, 53–56). The precise mechanism underlying the secretion of MIF, which lacks an N-terminal signal sequence, has remained enigmatic (34, 57). MIF has been reported to be associated with vesicles and exosomes (18, 19) and its release from stimulated monocytes is reduced by glyburide, which inhibits ATP-binding cassette transporter-dependent secretion (5).

We used a yeast two-hybrid approach to identify intracellular proteins that interact with MIF. The specificity of our screen was confirmed by the finding that 93% of the interacting clones were MIF itself, which associates into a noncovalent homotrimer (35, 36). However, we also identified a cDNA clone encoding the carboxyl-terminal 260 aa of the Golgi-associated protein p115. Identification of p115 as an interacting partner for MIF was confirmed by in vitro binding assays and endogenous coinmunoprecipitation. The competition binding assay using recombinant MIF and p115 protein suggests that no other proteins are necessary for the binding of the two proteins in vitro. Nevertheless, the competition curve levels at 50–60%, indicating that additional protein interactions may occur in vivo.

The immunofluorescent microscopy studies show that MIF is present in large cytoplasmic pools which disperse toward the periphery upon inflammatory stimulation. Similarly, p115 is also partially redistributed upon LPS stimulation from the Golgi toward the plasma membrane. It remains possible that LPS stimulation induces additional unknown proteins, which in turn might be necessary for the interaction between MIF and p115 and its export out of the cell.

Because the cellular depletion of p115 reduces stimulated MIF export, p115 may be essential for the transport of MIF from the perinuclear ring to the plasma membrane and then out of the cell. This pathway for release also is supported by the data demonstrating the accumulation of MIF and p115 in the supernatants of stimulated cells.

The partial depletion of p115 by the RNAi technique used in this study neither affected the structural morphology of the Golgi nor reduced the export of ER-dependent proteins such as TNF-α, IL-6, and FGF-4. The release of the unconventionally secreted protein, IL-1β, which occurs by vesicles (13), and FGF-2, which translocates across the plasma membrane (15), also were not affected by p115 depletion. That p115 is necessary for the release of MIF but not other cytokines, whether conventionally or unconventionally secreted, suggests that p115 has a specific role in MIF export. It also is possible that p115 mediates a generalized secretory response by activated monocytes/macrophages that results in the release of a p115 macromolecular complex containing numerous proteins, of which MIF is one. Further elucidation of both the signals and the protein-protein interactions underlying this export pathway may be informative and enhance our understanding of the acute secretory response of activated monocytes/macrophages.

That MIF is cosecreted with p115 was affirmed by the finding that the small molecule MIF inhibitor 4-IPP reduces the release of both proteins from monocytes/macrophages. These data identify 4-IPP as a potentially selective inhibitor of the p115/MIF secretory pathway. 4-IPP covalently modifies the MIF amino terminus (23) and it alters the protein’s conformational integrity so as to increase its binding interaction with p115. These results indicate that a “native” MIF-p115 interaction is essential for the efficient release of MIF from cells. A non-native MIF-p115 interaction may disrupt trafficking and downstream protein interactions that are necessary for efficient MIF release. 4-IPP also provides proof-of-concept for the possibility of reducing MIF-dependent responses in the earliest
phase of cell activation by interfering with MIF’s cytoplasmic re-lease. Inhibitors such as 4-IPP may be attractive in clinical appli-cation in those settings, such as severe inflammation, in which rapid intervention is desired to prevent the initiation of a tissue-damaging cytokine cascade (58). We note that our findings do not exclude additional actions for 4-IPP with respect to inhibiting MIF binding with its cell surface receptor or with other effector pro-teins. Whether p115 is involved exclusively in the stimulated ex-port of MIF or also plays a role in the constitutive release of MIF under basal conditions remains to be answered. The observation that 4-IPP inhibits MIF secretion under basal and stimulated con-ditions indicates a role for p115 in the constitutive release, but we were unable to observe a significant influence of genetic p115 depletion on MIF release under nonstimulated conditions.

In summary, these data describe a novel function for the Golgi-associated protein p115 in the mediation of MIF unconven-tional secretion. Pharmacologic targeting of the MIF-p115 interaction may offer a powerful approach for inhibiting innate immune responses by interfering with the secretion of the up-stream cytokine, MIF.

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Disclosures

The University of Louisville has applied for a patent describing the po-tentiation of the MIF inhibitor 4-IPP.

References


