FEBS 29368 FEBS Letters 579 (2005) 1693–1701

Binding of JAB1/CSN5 to MIF is mediated by the MPN domain but is independent of the JAMM motif

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Received 10 December 2004; revised 7 January 2005; accepted 31 January 2005

Available online 22 February 2005

Edited by Felix Wieland

Abstract **Macrophage migration inhibitory factor (MIF) binds to c-Jun activation domain binding protein-1 (JAB1)/subunit 5 of COP9 signalosome (CSN5) and modulates cell signaling and the cell cycle through JAB1. The binding domain of JAB1 responsible for binding to MIF is unknown. We hypothesized that the conserved Mpr1p Pad1p N-terminal (MPN) domain of JAB1 may mediate binding to MIF. In fact, yeast two hybrid (YTH) and in vitro translation/coimmunoprecipitation (CoIP) analysis showed that a core MPN domain, which did not cover the functional JAB1/MPN/Mov34 metalloenzyme (JAMM) deneddylase sequence, binds to MIF comparable to full-length JAB1. YTH and pull-down analysis in conjunction with nanobead affinity matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry demonstrated that MIF(50–65) and MPN are sufficient to mediate MIF–JAB1 interaction, respectively. Finally, endogenous CoIP of MIF–CSN6 complexes from mammalian cells demonstrated that MPN is responsible for MIF–JAB1 binding in vivo, and, as CSN6 does not contain a functional JAMM motif, confirmed that the interaction does not require JAMM.**

Keywords: Macrophage migration inhibitory factor; Cytokine; c-Jun activation domain binding protein-1; COP9 signalosome; Mpr1p Pad1p N-terminal domain

1. Introduction

c-Jun activation domain binding protein-1 (JAB1) is the subunit 5 of the COP9 signalosome (CSN). CSN was initially identified in plants, where it is involved in the suppression of light-dependent development [1]. CSN consists of 8 subunits; accordingly, JAB1 is alternatively termed subunit 5 of COP9 signalosome (CSN5). CSN is widely conserved between species and was also identified in eukaryotes, including human cells [2–4]. The 8 subunits of CSN share sequence homology with subunits of the 19S proteasome lid and with those of the translation initiation complex eIF3 [5]. Homology primarily comes from the similarity of the so-called MPN (Mpr1p Pad1p N-terminal) domain of CSN5 and CSN6, and from the PCI (proteasome, COP9, eIF3) domain of subunits 1–4 and 7–8 [6].

In mammalian cells, CSN is involved in the regulation of protein stability by cooperating with the 26S proteasome. For example, CSN has been implicated in the regulation of degradation of the transcription factors p27, p53, Id1, and Id3 [7–9]. CSN-mediated phosphorylation followed by ubiquitination appears to constitute a critical intermediate step during these processes. The mechanism by which the CSN contributes to ubiquitin-proteasome-mediated protein degradation is complex and has not yet been finally resolved [4]. CSN exhibits a metalloprotease/isopeptidase activity centered around its subunit JAB1/CSN5. CSN interacts with cullin-based E3 ubiquitin ligases and it has been established that through its isopeptidase activity, CSN removes the post-translational modification of a ubiquitin-like protein, neural presursor cell expressed developmentally down-regulated 8 (Nedd8)/Rub1, from the cullin component of SCF ubiquitin E3 ligase [4,10]. Accordingly, CSN has a deneddylation activity. It is currently believed that CSN, through its deneddylation activity, promotes cullin-dependent proteolysis in vivo [4,11]. The deneddylation activity was mapped to the JAB1/MPN/Mov34 metalloenzyme (JAMM) sequence motif of JAB1/CSN5 and deneddylation of neddylated cullin 1 by CSN is dependent on the presence of JAB1 [10]. The JAMM motif consists of five polar residues that resemble the active site residues of hydrolytic enzyme classes, particularly that of metalloproteases; the prototypical JAMM consensus sequence is EX_nHS/THX_7SXXD [10,12]. The His and Asp residues of this conserved motif coordinate a zinc ion. Within the CSN subunits, the JAMM motif, which is also termed MPN+ motif, is only present in JAB1/CSN5, but JAMM is found in certain other MPN domain-bearing proteins outside the CSN, including the 19S proteasome lid subunit POH1/Rpn11 [12–14]. Whereas JAB1/CSN5 is involved in deneddylation, POH1/

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Abbreviations: C60SMIF, MIF mutant with Cys60 exchanged for Ser; CALC, Cys–Ala–Leu–Cys motif; CSN, COP9 signalosome; CSN5, subunit 5 of COP9 signalosome; IVT, in vitro transcription/translation; FCS, fetal calf serum; JAB1, c-Jun activation domain binding protein-1; JAMM, JAB1/MPN/Mov34 metalloenzyme; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; MIF, macrophage migration inhibitory factor; MPN, Mpr1p Pad1p N-terminal; Nedd8, neural presursor cell expressed developmentally down-regulated 8; P2AMIF, MIF mutant with Pro2 exchanged for Ala; rMIF, recombinant human MIF; YTH, yeast two hybrid

0014-5793/$30.00
doi:10.1016/j.febslet.2005.01.080

Rpn11 catalyzes the removal of ubiquitin itself. Thus, the JAMM motif specifies a catalytic center that defines a family of zinc-dependent proteases that remove ubiquitin or ubiquitin-like proteins from components of the ubiquitin-proteasome degradation system.

JAMM stands for 'JAB1/MPN/Mov34 metalloenzyme', indicating that the JAMM motif is located within the conserved MPN/Mov34 domain of JAB1/CSN5. The precise functions of MPN domains are unknown, but they are necessary for proper interactions between subunits of the corresponding protein complexes. MPN domain proteins in eukaryotes include CSN5, CSN6, Rpn8/S12, Rpn11/POH1, AMSH, and C6.1A [6,12]. The MPN domain spans up to 120–140 amino acid residues and is predicted to have an α/β structure. It is typically present in the N-terminal part of a protein. A core MPN region containing the three similarity peaks I, II and III covers ~90 residues [6,15]. Recently, the 121 amino acid residue MPN domain-only protein AF2198/AfJAMM was identified and crystallized from an archaebacterium [16,17]. The obtained structure overall confirmed the predicted MPN structures and confirmed JAMM as a functional metalloprotease site.

That the MPN domain could serve as a conserved protein–protein interaction module in mammalian cells, was recently demonstrated experimentally. Tomoda et al. [18] showed that the N-terminal half of JAB1/CSN5 (residues 1–190 including the MPN domain) mediates the binding of JAB1 to the cell cycle inhibitor p27. Similarly, DNA topoisomerase IIα interaction with JAB1 is dependent on the MPN domain of JAB1, as a deletion mutant of JAB1 missing sequence region 55–190 does not bind to JAB1, while a C-terminal mutant spanning residues 1–190 strongly interacts [19]. Both for p27 and DNA topoisomerase IIα, MPN-target protein interaction is functionally important, regulating, among other effects, p27 and DNA topoisomerase IIα degradation.

To date, more than 20 proteins have been identified to interact with JAB1/CSN5. Among these proteins are other CSN subunits that are in direct contact with JAB1 as well as target proteins of the ubiquitin-proteasome degradation machinery. However, JAB1 functions may reach beyond a role in protein degradation. Accordingly, JAB1 (and the CSN) has been implicated in the regulation of signal transduction pathways and JAB1-interacting proteins include components of cell signaling pathways, such as the β2-integrin LFA-1 and the cytokine macrophage migration inhibitor factor (MIF) [20].

MIF is an evolutionary conserved 12.5 kDa protein that in humans functions as a pleiotropic cytokine with a mostly pro-inflammatory spectrum of action in the host immune response. As such, MIF is a critical mediator of a number of immune and inflammatory conditions (reviewed in [21]), including bacterial septic shock [22,23], rheumatoid arthritis [24], atherosclerosis [25], and tumorigenesis [26]. MIF is unique among cytokines, as it also has functions outside the immune system (reviewed in [27]) and can act as a glucocorticoid antagonist and endocrine factor [23,28].

Regulation of immune cell activity by MIF is at least in part mediated through CD74. CD74 is the invariant chain (Ii) of the major histocompatibility complex II (MHC II), but is also expressed independently of MHC II on the plasma membrane. Surface CD74 serves as a membrane receptor for MIF and is involved in the stimulation of cell proliferation and ERK MAPK signaling by MIF [29].

Of note, an intracellular role of MIF could be related to its catalytic properties, i.e., its thiol-protein oxidoreductase (TPOR) activity, that is dependent on a Cys–Ala–Leu–Cys (CALC, CXXC) motif [30–32]. In addition, the TPOR activity of MIF could play a role in MIF-mediated inhibition of apoptosis and regulation of monocyte/macrophage activation [31,33,34]. A MIF-derived TPOR motif-spanning peptide exhibits MIF-like TPOR activity and mimics MIF cell-regulatory functions [34].

Intracellular functions of MIF also encompass its binding to JAB1/CSN5. Both endogenous intracellular MIF and extracellular MIF following endocytosis can interact with JAB1 and MIF can regulate certain of the cellular activities of JAB1 [35]. Through binding to JAB1, MIF antagonizes stimulation of activator protein-1 (AP-1) activity by JAB1. MIF inhibits both JAB1-induced c-Jun-N-terminal kinase (JNK) activity and AP-1 transcriptional activation. Also, the JAB1-mediated overcoming of cell cycle arrest and JAB1-induced p27 degradation is counter-regulated by MIF. Accordingly, MIF stabilizes p27 levels in G1-arrested cells [35]. MIF–JAB1 interaction appears to serve a critical role during inflammatory processes, as such complexes were specifically observed in atherosclerotic plaque tissue [25] and pituitary tumors [36].

On the side of MIF, sequence region 50–65 is important for the interaction with JAB1, as MIF peptide 50–65 competes with full-length MIF for JAB1 binding [35], and because this MIF-agonistic peptide directly binds to full-length JAB1 [34]. While the peptide spans the CXXC motif of MIF, it appears that the cysteine residues themselves are not necessary for mediating the interaction with JAB1, as a bis-serine variant peptide also competes with recombinant human MIF (rMIF) and also directly binds to JAB1 [34,35]. In contrast, it has not been addressed which domain or sequence region of JAB1 contributes to MIF–JAB1 interaction.

We hypothesized that the MPN module of JAB1 which was reported previously to be important for protein–protein interactions, could mediate the binding of MIF to JAB1. Thus, in the current study we devised a core MPN domain and examined by yeast two hybrid (YTH) and coimmunoprecipitation (CoIP) analysis, whether this domain participates in the interaction. To further define the MIF–JAB1 binding event, the CXXC-spanning MIF peptide 50–65 and its bis-serine variant were included in the investigation, and the potential physiological relevance of MPN-mediated MIF–JAB1 interaction was addressed by endogenous interaction studies in vivo.

2. Materials and methods

2.1. Cell culture and miscellaneous reagents

General cell culture reagents such as media, supplements, antibiotics, and serum were from Gibco Invitrogen Corporation (Karlsruhe, Germany). Human epithelial kidney cells (HEK293) were bought from the German Society for Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and were maintained by routine protocols in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum (FCS), 1% penicillin–streptomycin, and 5 mM of L-glutamine. Cells were subcultured 2–3 times a week and passages 5–15 were used for the experiments. All cell culture experiments were performed at 37 °C in a humidified incubator with 5% CO_2.

Miscellaneous reagents such as chemicals and salts were from Sigma–Aldrich Chemicals (Taufkirchen, Germany). All reagents were of the highest grade commercially available.

2.2. Yeast two hybrid screening assay

We previously identified the interaction between MIF and JAB1 by YTH screening [35]. Applying an essentially identical YTH system (Matchmaker 2; Clontech, Heidelberg, Germany), the coding region of the human MIF gene was fused in-frame to the GAL4 DNA binding domain (GAL4-BD) using the pAS2-1 vector as described previously [35]. The resulting bait plasmid, pMIF-BD, was used to examine the interaction between human MIF and the MPN domain of JAB1 by YTH screening essentially following the manufacturer's protocol. A core region of the MPN domain covering amino acids 53–142 but not spanning the full JAMM motif was fused in-frame to the GAL4 DNA activation domain (GAL4-AD) using the pACT2 vector (pMPN-AD). Cloning of the full-length human JAB1 gene into pACT2 was described previously [35]. The MPN insert was generated from this construct using an EcoRI site-containing forward primer 5′-CGG AAT TCA TGA AGT ACT GCA AAA TCT CAG CAT TG-3′ and the SacI site-containing reverse primer 5′-GGA GCT CCT AAT AGC CAG GGT GGC TAT GAT ACC AC-3′. For structure function analysis purposes, MIF mutants were also cloned into pAS2-1. The mutants used were the tautomerase-dead mutant MIF mutant with Pro2 exchanged for Ala (P2AMIF) and the oxidoreductase-dead mutant MIF mutant with Cys60 exchanged for Ser (C60SMIF) [37]. Mutant constructs in pAS2-1 were generated by amplifying the mutant sequences from their corresponding pET11b vectors, using the same cloning sites as for wild-type human MIF [35].

For cotransformations of the resulting bait and prey plasmids, pMIF-BD (or the mutated MIF variants) and pMPN-AD (or pJAB1AD), respectively, yeast strain CG1945 was used when testing for growth on His drop-out medium (Clontech) and strain Y190 was applied when testing for β-galactosidase activity. The following constructs were used as positive and negative controls for the (co)transformations: pCL1, positive control plasmid encoding for full-length wild-type GAL4 protein; pVA3-1/pTD1-1, cotransformed positive control plasmids encoding for a p53-BD fusion protein in pAS2-1 and a SV40 T-antigen-AD fusion protein in pACT-2, respectively. (Co)transformations and YTH screens were performed essentially as described by the manufacturer.

Selection for significant interactions was carried out on Trp− Leu− His− medium and we tested positive clones containing GAL4 DNA activation domain fusion proteins for β-galactosidase activity using the ONPG (*o*-nitrophenyl β-D-galactopyranoside, Roche Diagnostics, Mannheim, Germany) test. The ONPG test was performed essentially following the method of Miller, except that an optimized yeast cell lysis procedure using lyticase (5 U/μl in TE buffer, 200 μl/10 ml of cell suspension) was applied [38]. Briefly, the chromogenic substrate ONPG was added in excess. After incubation at 30 °C, the reaction was stopped by raising the pH to 11 to inactivate β-galactosidase. Product formation was determined spectrophotometrically and plotted in Miller units.

2.3. In vitro translation, coimmunoprecipitation and SDS–PAGE/Western blot analysis

CoIPs were performed both following in vitro transcription/translation (IVT) reactions and from HEK293 kidney epithelial cell lysates.

For CoIPs from IVT lysates, rMIF and radioactively labeled MPN were used. Biologically active natively folded rMIF was prepared as described previously [35]. The MPN cDNA was amplified from pCIneo-JAB1 [35] using forward primer 5′-CTA GCT AGC ATG AAG TAC TGC AAA ATC TCA GCA TTG-3′, containing an NheI site, and reverse primer 5′-CGG GAT CCC TAA TAG CCA GGG TGG CTA TGA TAC CAC-3′, containing a BamHI site, and was fused into the pET11b vector (Novagen-Calbiochem, Heidelberg, Germany). For IVT, the in vitro PBS Kit™ from RiNA GmbH (Berlin, Germany) was used. Gene expression in this *Escherichia coli*-derived cell-free protein biosynthesis system is under control of the T7 promoter. Expressed target protein was radiolabeled by incorporation of ^{35}S-methionine. 30 μl of the IVT lysate was diluted with phosphate-buffered saline (PBS, pH 7.4), and rMIF added to give a final volume of 1045 μl. The incubation was divided and equal portions used for the CoIP and control incubations. The solution was incubated by end-over-end rotation for 1 h at room temperature. To pull-down rMIF–^{35}S–MPN complexes, a polyclonal rabbit anti-MIF antibody (Ka565) was added and the incubation continued for 1 h at room temperature by end-over-end rotation. The control was incubated identically except that the anti-MIF antibody was omitted. Protein A sepharose (Amersham-Pharmacia, Freiburg, Germany) was added for 2 h (200 μl slurry blocked overnight with 5% dry-milk powder; room temperature; end-over-end rotation). Washes (4 times) were performed with 0.1 × HNT buffer (25 mM HEPES, pH 7.2, 1.5 mM $MgCl_2$, 2 mM EDTA, 3 mM DTT, 0.5% Triton X100; end-over-end rotation for 5 min each). ^{35}S–MPN coimmunoprecipitated with rMIF was then revealed by SDS–PAGE electrophoresis in a 18% gel and, upon drying of the gel, exposure of an X-ray screen.

Binding of MIF to CSN6 was examined by CoIP analysis of endogenous complexes from HEK293 cells. To be able to directly compare the result of the MIF–CSN6 CoIP with a recently optimized CoIP of MIF with JAB1 from HEK293 cells expressing both endogenous JAB1 and ectopically overexpressed myc-tagged JAB1, MIF–CSN6 CoIPs were performed from HEK293 cells that were stably mock-transfected with the pCIneo vector (Lue et al., submitted). Briefly, stable transfections of HEK293 cells were performed as follows: cells were transfected with the linearized pCIneo-mycJAB1 and pCIneo vectors using the SuperFect transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Cells were incubated with 800 μg/ml G418 for 2 weeks. Positive clones potentially carrying the myc-tagged JAB1 sequence stably integrated in their genome were further selected through treatment with 200 μg/ml of G418 for another 8 weeks. Positive clones were identified by Western blotting analysis.

HEK293neo cells were lysed in low stringency CoIP buffer (50 mM Tris–HCl, pH 7.5, 15 mM EGTA, 100 mM NaCl, 0.1% Triton X100, 10% glycerin, 25 mM sodium fluoride, 1 mM DTT, 1 × proteinase inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), 1 mM sodium azide), 2 μg polyclonal rabbit anti-CSN subunit 6 antibody (Biotrend, Köln, Germany) added, and the incubation continued for 1.5 h at 4 °C. Milk powder-blocked (5% in PBS) protein A sepharose beads (50 μl slurry) were washed with PBS and CoIP buffer and added to the incubation. After 1 h at 4 °C, beads were washed 3 times with CoIP buffer. Bound protein was eluted by adding 1× reducing NuPAGE sample buffer (SLB-DTT) and boiling. Precipitated proteins were separated in 4–12% NuPAGE gels (Invitrogen, Karlsruhe, Germany) and transferred to nitrocellulose. Coimmunoprecipitated endogenous MIF was revealed by Western blotting using a polyclonal rabbit anti-MIF antibody (Ka565) as described previously [35]. For control, blots were redeveloped for CSN6 using the above antibody. For comparison of MIF–CSN6 with MIF–JAB1 binding, CoIPs of endogenous MIF–JAB1 complexes were performed from HEK293mycJAB1 cells by a similar protocol, except that the mouse monoclonal anti-JAB1 antibody 8H8.5 from Genetex (San Antonio, TX, USA) and protein G sepharose beads were used for the CoIP. In these experiments, complexes of overexpressed mycJAB1 and MIF were also analyzed by co-adding an anti-myc 9E10 mouse monoclonal antibody (Roche Diagnostics), but the resulting bands were not used for the comparison. Detection of bands was achieved by ECL chemoluminescence. Incubation with the corresponding peroxidase-conjugated secondary antibody was performed at room temperature for 2 h at a dilution of 1:10 000 in blocking buffer. As ECL reagent, SuperSignal West Dura (Pierce-Perbioscience, Bonn, Germany) was used. Staining was measured by the LAS-3000 imager (Raytest, Isotopenmessgerät GmbH, Straubenhardt, Germany).

2.4. Nanobead pull-down and mass spectrometry analysis

To test the interaction of the MPN domain with MIF peptide MIF(50–65), silica-streptavidin-nanobeads (SAv-beads) and biotin-MIF(50–65) were applied. A bis-serine variant of biotin-MIF(50–65), with the two cysteine residues of the CXXC motif of MIF exchanged for serine, was used for comparison. The synthesis of biotinylated MIF(50–65) and biotinylated bis-serine-MIF(50–65) has been described previously [34]. SAv-nanobeads were developed by the Tovar group as described previously [39] and had a biotin binding capacity of 800 pmol/mg beads. For interaction and pull-down, 0.5 μl of a 1 mg/ml biotin-MIF(50–65) solution (250 pmol in 20 mM sodium phosphate buffer, pH 7.2) and 10 μl of an IVT reaction expressing non-radioactively labeled MPN domain (see above; corresponding to an estimated amount of ~95 pmol) were added to a dispersion of 250 μg SAv-beads in 250 μl PBS (200 pmol Biotin binding capacity). PBS was added to 500 μl and the reaction incubated for 3 h at room temperature under gentle shaking conditions. Control incubations contained identical amounts of beads and MPN but no biotin-MIF(50–65).

Upon immobilization, beads were washed with 2×1 ml PBS/0.1% Triton X100 (10 min shaking at 5 °C each), with 2×1 ml PBS (10 min shaking at 5 °C each), and with 1×1 ml double-distilled water (10 s vortexing). After the final washing step, the pellet was resuspended in 10 μl water and this solution used directly for the nanobead affinity matrix-assisted laser desorption ionization-MS (MALDI-MS) analysis.

For the mass spectrometry measurements, a linear LD-time of flight (LD-TOF) system (HP G 2025A from GSG Mess-und Analysengeräte GmbH, Bruchsal, Germany) was used. The instrument was equipped with a time lag focusing (TLF) unit and a digital oscilloscope (LeCroy 9350) for data acquisition. Measurements were performed at 20 kV acceleration power and under optimized TLF conditions for a center mass of 10 445 Da using the positive ion mode. For the analyses, a so-called sandwich preparation was used: 0.5 μl sinapinic acid (20 mg/ml in 99% acetone) was added to a MALDI target and air-dried. Subsequently, 1–3 μl bead suspension was added in 1 μl steps onto the dried matrix layer and dried under vacuum (Hewlett-Packard Sample Prep Accessory). Onto the top-most layer, another 0.5 μl sinapinic acid (20 mg/ml 50% acetonitrile/0.1% trifluoroacetate (TFA)) was pipetted and the mixture again air-dried. The measurements were performed under ultra-vacuum conditions (5×10^{-7} Torr). For external calibration, MALDI-MS analyses with the standard proteins horse cytochrome C and horse myoglobin (Sigma–Aldrich) were performed under the same conditions.

3. Results

3.1. The MPN domain of JAB1 binds to MIF as assessed by YTH and protein–protein interaction analysis in vitro

To test whether the MPN domain of JAB1 was necessary for MIF–JAB1 interaction, a 'core' MPN domain was cloned. 'Core' MPN (coreMPN) spanned residues 53–142 of human JAB1, therefore encompassing a main structural core of MPN including the three prominent similarity peaks I, II and III according to Asano et al. [15]. CoreMPN contained 2α-helices and 3β-strands according to the secondary structure prediction method of Garnier et al. [40] and aligned reasonably well with the MPN domain protein AF2198 of *A. fulgidus* [16] as assessed by the NCBI conserved domain search blast program (CD alignment; score: 38.4 bits). CoreMPN did not cover the JAMM motif residues Ser150 and Asp153 and thus did not contain a functional JAMM metalloprotease site [17].

First, the core MPN sequence was cloned into the pACT2 vector fused to the GAL4 DNA activation domain (MPN-AD) and was used as the prey sequence in a YTH analysis with full-length human MIF as bait (MIF-BD). Three independent clones that grew following nutritional selection on Leu−/Trp− CG1945 yeast cells (MIF/MPN1, MIF/MPN2. MIF/MPN3), were further tested for growth on His drop-out medium (Leu−/Trp−/His−). All clones were found to grow on this selection medium comparable to the positive control p53/SV40. In contrast, combining MIF-BD with GAL4-AD and Lamin-BD with MPN-AD did not result in colony growth on triple drop-out medium (Fig. 1A). YTH analysis by the liquid culture ONPG assay in Y190 yeast cells was then applied to confirm the observed MIF–MPN interaction and to quantitate it. Fig. 1B shows that the MIF–MPN interaction was weaker than that observed for p53–SV40. This was expected, since the MIF–JAB1 interaction was previously found also to be weaker than that between p53 and SV40 [35]. Furthermore, additional analysis by the ONPG assay demonstrated that the MIF–MPN interaction was essentially identical in strength to that observed between MIF and full-length JAB1, indicating together that coreMPN is sufficient for mediating the interaction between MIF and JAB1 (Fig. 1C).

To confirm the observed binding between MPN and MIF at the protein level, CoIP analysis was performed. Since we were unable to recombinantly express and purify MPN in sufficient amounts from *E. coli*, radioactively labeled coreMPN was produced by IVT and incubated together with bacterially expressed, purified, biologically active rMIF. Potential ^{35}S–MPN–rMIF protein complexes were immunoprecipitated with anti-MIF antibody and coprecipitated ^{35}S–MPN analyzed by gel electrophoresis. Immunoprecipitation with anti-MIF led to the detection of an MPN band that was markedly stronger than that detected following a control incubation without anti-MIF antibody, confirming that specific ^{35}S–MPN–rMIF protein complexes had formed (Fig. 2).

Together with the YTH analyses, this indicated that coreMPN mediates the binding of JAB1 to MIF and, because coreMPN does not contain a complete JAMM motif, suggested that MIF–JAB1 binding was independent of JAMM.

3.2. MIF sequence 50–65 mediates binding to MPN

The sequence stretch 50–65 of MIF was previously shown to compete with rMIF for JAB1 binding [35] and can directly bind to JAB1 [34]. In addition, MIF(50–65) exhibits certain MIF-agonistic effects [34]. We therefore asked whether MIF(50–65) was able to bind to the MPN domain of JAB1. Biotinylated MIF(50–65) was prepared and incubated with coreMPN that was produced by IVT. Formed complexes were then subjected to pull-down analysis using the SAv-nanobeads and coprecipitated MPN protein detected by mass spectrometry. Fig. 3 shows that MIF(50–65) markedly interacted with coreMPN (upper/red spectrum), whereas in a control incubation containing the beads but no biotin-MIF(50–65) (bottom/black spectrum) no MPN was detected by the MALDI-MS analysis. Previous studies had shown that the binding of MIF(50–65) to JAB1 is not dependent on the presence of the two cysteine residues at positions 57 and 60, as a corresponding bis-serine variant of MIF(50–65) bound to JAB1 similar to the wildtype peptide [34]. We therefore compared the binding between biotin-MIF(50–65) and coreMPN with that of biotin-bis-serine-MIF(50–65). MALDI-MS analysis revealed that the variant peptide also bound to coreMPN (Fig. 3, middle/blue spectrum). Binding of the variant peptide appeared to be weaker than that of the wildtype peptide, but such a quantification is unsafe, since MALDI-MS analysis is only a semi-quantitative method.

Binding of MIF(50–65) and its serine variant peptide to coreMPN further confirmed that MPN is sufficient to mediate binding of JAB1 to MIF, but also showed that the redox-active cysteines that are part of the CXXC sequence stretch, are not required for the interaction between MPN and MIF. YTH analysis using a CXXC variant of MIF, C60SMIF, instead of wildtype MIF yielded no positive clones on the triple-drop-out medium. It is currently unclear, whether lack of a positive YTH result for C60SMIF is due do a lack of interaction with MPN or whether it is due to protein misfolding/aggregation and/or lower expression levels of this mutant compared to the wildtype protein. To further extend the structure function analysis of the MIF–MPN interaction, we next asked whether the sequence motif

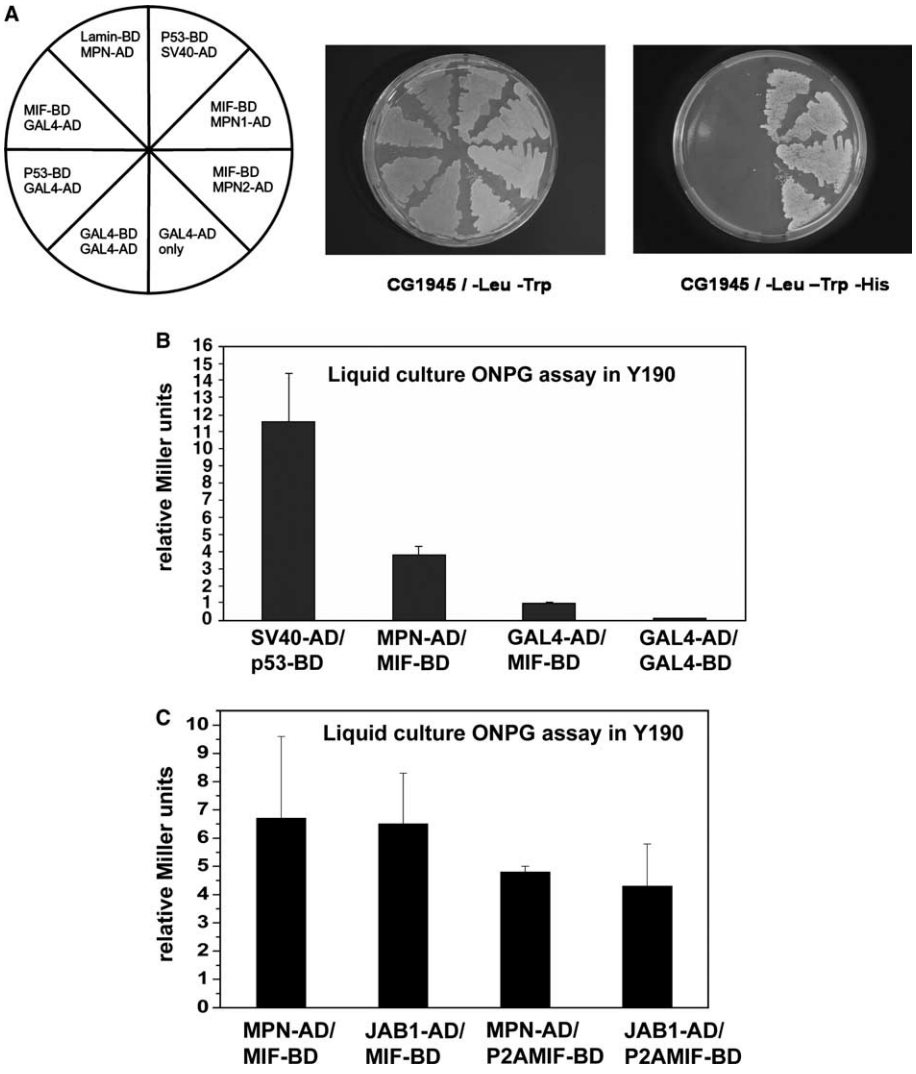

Fig. 1. Yeast two hybrid (YTH) analysis of the MIF–MPN interaction. (A) MIF interacts with the core MPN domain of JAB1/CSN5 in the YTH assay. Growth of yeast transformants (strain CG1945) coexpressing wildtype human MIF and coreMPN on nutritional selection medium. Left, overview of plate streaking profile; middle, Leu+ Trp+ transformants were streaked on media lacking leucine and tryptophan (CG1945/−Leu −Trp); right, transformants were streaked on triple-drop-out media (CG1945/−Leu −Trp −His). Only the positive control p53-BD/SV40-AD and the three MIF-BD/MPN-AD clones grow. (B) Quantification of the MIF–MPN interaction in the YTH system by the liquid media assay according to Miller. β-Galactosidase activity was determined by liquid media assay in strain Y190. Activity of the MIF-BD/MPN-AD samples is ~3× lower than that of p53-BD/SV40-AD, but significantly higher than that of the negative controls GAL4-AD/MIF-BD and GAL4-AD/GAL4-BD. (C) Quantitative comparison of the MIF–MPN interaction with that of MIF–JAB1 and P2AMIF–JAB1 and P2AMIF–MPN by YTH analysis. As in (B), the liquid media assay was applied. Values in (B) and (C) are means ± S.D. of 3 determinations. Statistics were calculated by the two-sided student's *t* test. The differences in (B) between MIF/MPN and the positive and negative controls were significant ($P < 0.05$). The differences between P2AMIF/MPN–wtMIF/MPN and P2AMIF/JAB1–wtMIF/JAB1 in (C) were not significant ($P > 0.05$).

responsible for the other enzymatic activity of MIF, the tautomerase activity, was required for the interaction. The Pro-2 residue is critical for MIF's tautomerase site and its relevance for the interaction between MIF and JAB1 has not been investigated. We thus checked, whether the tautomerase mutant P2AMIF was able to bind to JAB1 and MPN. Quantitative YTH analysis demonstrated that P2AMIF significantly bound to both JAB1 and MPN (Fig. 1C). Binding of P2AMIF to JAB1 and MPN was almost identical in strength, but was slightly weaker than that of wildtype MIF. However, this difference was not statistically significant. Together, this indicated that the tautomerase site around residue Pro-2 is not critical for the binding of MIF to JAB1 and MPN.

3.3. The MIF–MPN interaction occurs in vivo at endogenous protein concentrations

The MIF–JAB1 interaction was previously shown to occur in mammalian cells and to mediate modulation of JAB1 activity by MIF [35]. To test whether binding of MIF to the MPN domain occurs in a physiologically relevant environment such as in a mammalian cell, and thus is relevant for the interaction between MIF and JAB1 in vivo, we next investigated whether MIF was able to bind to an MPN domain-containing protein in the cell that was distinct from JAB1. CSN6, another subunit of the CSN, was elected as its MPN domain exhibits an extended homology to the JAB1 MPN domain. CoreMPN spans a major part of this homology region (Fig. 4A). The potential interaction between MIF and CSN6 was examined by CoIP

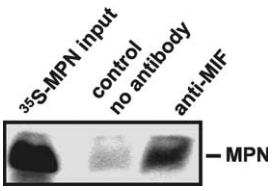

Fig. 2. Coimmunoprecipitation of protein complexes of rMIF and in vitro-translated MPN. Bacterially expressed purified recombinant MIF was incubated with ^{35}S-labeled coreMPN domain produced by in vitro transcription/translation, and protein complexes were immunoprecipitated by anti-MIF antibody (anti-MIF) in comparison to control incubations without antibody. Coprecipitated MPN was revealed by gel electrophoresis and autoradiography. As input control for the CoIP, 10% of the amount of ^{35}S–MPN used was electrophoresed for comparison.

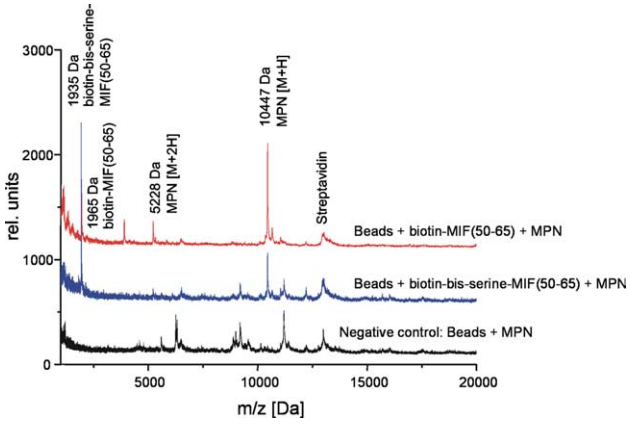

Fig. 3. Interaction of MIF peptide 50–65 with MPN as demonstrated by CoIP and nanobead affinity mass spectrometry. Biotin-labeled MIF(50–65) was incubated with in vitro-translated coreMPN and complexes precipitated by streptavidin nanobead affinity pull-down. For comparison, incubations were performed with the bis-serine variant of MIF(50–65) and for negative control, with beads alone. Precipitates were directly subjected to MALDI-MS as described in Section 2. Upper/red spectrum: incubation of biotin-MIF(50–65) with MPN; middle/blue: incubation of bis-serine variant of biotin-MIF(50–65) with MPN; bottom/black: control incubation without biotinylated peptide. MALDI-MS signals are given as relative units. In addition to the M + H peaks of MPN, the M + 2H peak of MPN, peaks for the biotinylated MIF peptides and streptavidin were detected as indicated.

between endogenous MIF and endogenous CSN6 using an anti-CSN6 antibody for CoIP. Fig. 4B demonstrates that a marked MIF band was coprecipitated by the anti-CSN6 antibody, whereas a control incubation with beads alone only led to a minor band due to some residual non-specific adsorption. This indicated that MIF–CSN6 protein complexes form intracellularly at physiological conditions and non-overexpressed endogenous concentrations of both components. At the same time, this analysis demonstrated that the MPN domain is sufficient in vivo to mediate MIF–JAB1 interaction and that a MIF–CSN6 interaction occurs in vivo. Comparison with a CoIP of endogenous MIF–JAB1 complexes revealed that formation of the MIF–CSN6 complexes was slightly weaker than that of the MIF–JAB1 complexes. Since CSN6 does not contain a functional JAMM motif within its MPN domain (Fig. 4A), endogenous formation of MIF–CSN6 complexes confirmed that the JAMM motif and JAMM-mediated isopeptidase activity were not necessary for binding of MIF to JAB1.

In summary, these data showed that the MPN domain independent of its JAMM function is sufficient to mediate MIF–JAB1 interaction in vitro and in vivo, that, on the side of MIF, sequence 50–65 accounts for the binding to MPN, and that MIF also binds to CSN6 in a MPN-dependent fashion.

4. Discussion

Macrophage migration inhibitory factor critically participates in the regulation of the host inflammatory response. Originally, MIF was thought to predominantly regulate cell migration, but today it is known that MIF-driven effects encompass a broad spectrum of typical cytokine-mediated effects on cell proliferation, cell apoptosis, cell cycle arrest, and transcriptional induction of a variety of genes [21]. Modulation of immune cell function by MIF is often proinflammatory and includes stimulation of cell proliferation and MAPK kinase signaling in a CD74-dependent manner [21,29].

On the other hand, it appears that MIF utilizes the JAB1-mediated signaling pathway to fine-tune an antiinflammatory cellular response [35,41]. Cell regulation by MIF through JAB1 is either initiated by preformed intracellular MIF or occurs upon endocytosis of extracellular MIF into target cells [35,42]. The details of both the CD74- and JAB1-mediated signaling pathways still need to be unraveled. As to the JAB1-mediated pathway, it will be important to understand the mechanism of MIF–JAB1 interaction, as for example, MIF binding to JAB1 could interfere with recognition by JAB1 of neddylated substrates or subcellular transportation and delivery to the CSN, which then might be responsible for further signal transduction regulatory processes [20,43]. While it has become evident that MIF sequence 50–65 participates in MIF–JAB1 interaction, it has been unknown which sequence region or domain of JAB1 is responsible for binding to MIF. Defining the interaction modules on both binding partners will be important to further characterize the mechanism of JAB1-mediated cell regulation by MIF.

In the current study, we have demonstrated that the MPN domain of JAB1, which was previously demonstrated to mediate the binding of JAB1 to p27 and topoisomerase IIα, is responsible both in vitro and in vivo for the binding of MIF to JAB1. Since we have used a core MPN domain that did not encompass the functional JAMM motif, it is apparent that the JAMM site and activity is not necessary for MIF binding. Binding of the MIF-agonistic peptide 50–65 and its bis-serine variant to MPN further indicated that sequence regions MIF 50–65 and JAB1 53–142 represent minimal regions enabling for an interaction between MIF and JAB1. Future structure activity studies will need to be performed to define the binding affinities between these modules and to examine whether longer or shorter regions will lead to an increase or decrease in the interaction.

Interaction between MIF and the core MPN region of JAB1 was first demonstrated by YTH analysis by both plate assay and the quantitative liquid media assay. Of note, the interaction between MIF and coreMPN was comparable to that between MIF and full-length JAB1 [35], indicating that the elected MPN region contained the minimal interaction region required. Nevertheless, since YTH screens can lead to false-positive results and because truncation of MPN might have

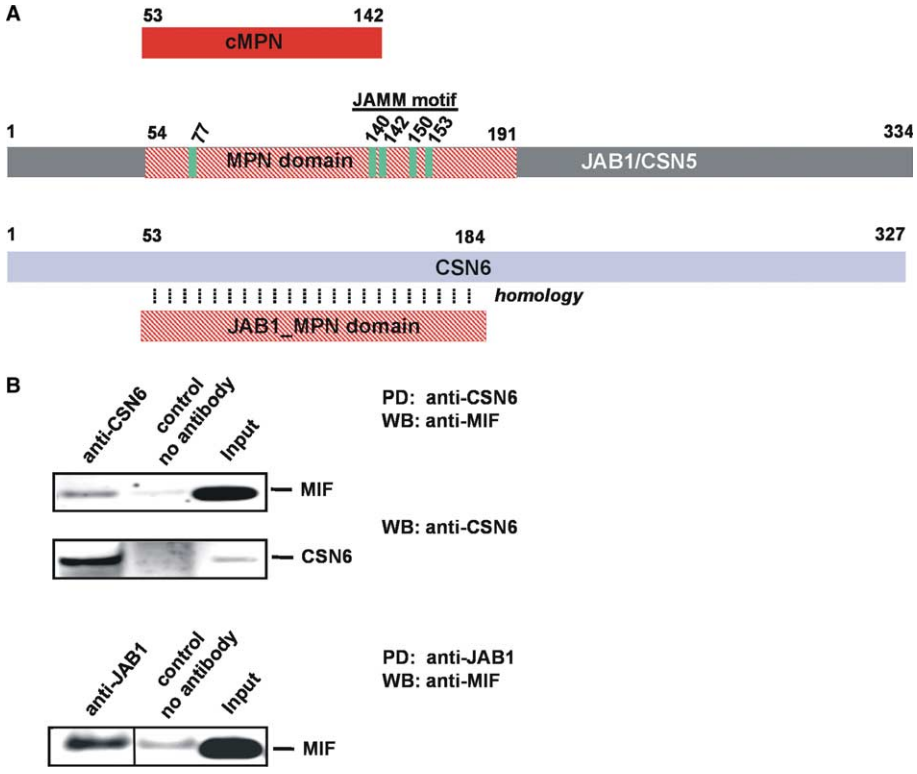

Fig. 4. Interaction of endogenous MIF with endogenous MPN in vivo and independency of the MIF–MPN interaction of the JAMM motif. (A) Scheme of the MPN domain of JAB1/CSN5 and homology with the core MPN domain used in Figs. 1–3 of this study and the MPN domain of CSN6. The core MPN domain (cMPN) spans JAB1 residues 53–142. For JAB1/CSN5, the positions of the residues forming the JAMM motif are indicated. The MPN domain of CSN6 does not contain a JAMM motif. (B) Endogenous coimmunoprecipitation of MIF–CSN6 complexes and comparison with MIFJAB1/CSN5 complexes. Top two panels: Immunoprecipitation of CSN6 complexes with anti-CSN6 antibody and detection of coprecipitated MIF by Western blot analysis (upper panel). For control, CoIPs were performed with beads alone. One-tenth of the CoIP incubation sample was co-electrophoresed as input-control. Blot was stripped after MIF detection and re-developed with anti-CSN6 to control for CoIP efficiency (middle panel). Lower panel: for comparison, endogenous MIF–JAB1 complexes were coimmunoprecipitated using anti-JAB1 antibody. Western blot analysis was performed as above.

led to a hydrophobic patch that non-specifically mediated interaction in the yeast system, MIF–MPN binding was confirmed by protein–protein interaction studies in vitro. These studies demonstrated that purified biologically active MIF formed specific complexes with in vitro-translated coreMPN. The previous studies investigating the role of MPN in JAB1 binding to p27 and topoisomerase IIα have applied the entire N-terminus of JAB1 spanning residues 1–190 and covering the JAMM site [18,19]. Our study demonstrates that for JAB1 interaction with MIF, a shorter sequence stretch is sufficient and that the JAMM site or activity is not needed. That MIF–MPN binding occurs physiologically in a mammalian cell was further shown by endogenous CoIP between MIF and another MPN-containing mammalian protein, the CSN component CSN6. As CSN6 does not contain a JAMM site, this experiment also provided in vivo evidence that MIF–JAB1 interaction is JAMM-independent.

Recently, thioredoxin (Trx) an enzyme and co-cytokine [44,45] that shares with MIF its TPOR catalytic activity and redox-based anti-apoptotic function [33,46] was found to bind to JAB1 and to regulate JAB1-mediated cell functions in a manner essentially identical to that of MIF [47], indicating that MIF and Trx might not only share redox-regulatory activities and cytokine-like functions, but might also interact with JAB1 in a similar manner. However, JAB1 binds to Trx through its C-terminal region independent of MPN [47]. Thus, MIF and Trx both regulate JAB1 functions in a similar fashion, but the initial molecular binding event is different between these two mediators. It will be interesting to investigate whether the binding of MIF to JAB1 can nevertheless interfere with JAB1–Trx interaction or vice versa, or whether the respective interactions represent independent processes.

Binding of MIF(50–65) to MPN confirmed our prior observations that the sequence region around the CXXC motif of MIF is critical for interaction with JAB1 [35]. As also the bis-serine variant of this peptide bound to MPN, these studies in addition confirmed that it is the sequence region itself independent of the presence of the two Cys residues that confers binding to JAB1 [34]. Although we did not observe an interaction for the C60SMIF mutant in the YTH assay, the latter conclusion is nevertheless likely to hold true. C60SMIF has previously been shown to bind to JAB1 [35], and since it was also demonstrated that C60SMIF has a high tendency to aggregate when expressed in cells not containing endogenous MIF such as in *E. coli* [37], we suggest that the lack of interaction is due to aggregation/misfolding/degradation processes. This will need to be investigated further in the future. In line with the notion that the sequence region around the CXXC motif is important for MIF–MPN binding, we observed that P2AMIF, a MIF variant with a mutation in the N-terminal

isomerase site of MIF, bound to JAB1 and MPN comparably to the wildtype protein. Thus, the N-terminal site does not appear to be involved in the interaction.

In conclusion, we have identified a core MPN module of JAB1 as the binding region for MIF. MIF binding to JAB1 is JAMM-independent and occurs in vivo. The data further suggest that MIF not only binds to the CSN component JAB1/CSN5 but can also interact with CSN6. Since the MIF–CSN6 interaction is somewhat weaker than that between MIF and JAB1/CSN5, it may be implied that JAB1 is the major interaction partner of MIF in the cell, but that additional weaker interactions occur with other MPN domain-containing proteins. The precise interplay between these binding partners and the corresponding biological effects are likely to be critically involved in a variety of cell functions and will thus be worthwhile of exploring.

Acknowledgments: We thank A. Kapurniotu for synthesizing the biotinylated MIF peptides and R. Kleemann for assistance with the yeast two hybrid analysis. We are grateful to H. Lue, H. Brunner and K. Borchers for helpful discussions. This work was supported by Grant No. SFB 542/TP-A7 of the Deutsche Forschungsgemeinschaft (DFG) to J.B. and by the Bundesministerium für Bildung und Forschung (BMBF; proteomics program) Grant No. FKZ:0312834F to J.B. and G.E.M.T.

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