A 16-Residue Peptide Fragment of Macrophage Migration Inhibitory Factor, MIF-(50–65), Exhibits Redox Activity and Has MIF-like Biological Functions*

Macrophage migration inhibitory factor (MIF) is a cytokine that participates in the host inflammatory response. A Cys-Xaa-Xaa-Cys (CXXC)-based thiol-protein oxidoreductase activity of MIF is associated with certain biological functions. Peptides spanning the CXXC region of thiol-protein oxidoreductases retain some biochemical properties of the full-length protein. We report on the characterization of CXXC-spanning MIF-(50–65) and its serine variant, C57S/C60S-MIF-(50–65). Following disulfide-mediated cyclization, MIF-(50–65) adapted a β-turn conformation comparable with that of β-turn-containing cyclo-57,60-[Asp57,Dap60]-MIF-(50–65). MIF-(50–65) had a redox potential $E^\circ$ of ~0.258 V and formed mixed disulfides with glutathione and cysteine. MIF-(50–65) but not C57S/C60S-MIF-(50–65) had oxidoreductase activity in vitro. Intriguingly, MIF-(50–65) exhibited MIF-like cellular activities. The peptide but not its variant had glucocorticoid overriding and proliferation-enhancing activity and stimulated ERK1/2 phosphorylation. MIF-(50–65) and its variant bound to the MIF-binding protein JAB1 and enhanced cellular levels of p27Kip1. As the peptide and its variant were endocytosed at similar efficiency, sequence 50–65 appears sufficient for the JAB1-related effects of MIF, whereas other activities require CXXC. Cyclo-57,60-[Asp57,Dap60]-MIF-(50–65) activated ERK1/2, indicating that CXXC-dependent disulfide and β-turn formation is associated with an activity-inducing conformation. We conclude that CXXC and sequence 50–65 are critical for the activities of MIF. MIF-(50–65) is a surprisingly short sequence with MIF-like functions that could be an excellent molecular template for MIF therapeutics.

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§§ To whom correspondence may be addressed: Laboratory of Bioorganic and Medicinal Chemistry, Institute of Biochemistry, University Hospital RWTH Aachen, D-52074 Aachen, Laboratory of Bioinorganic and Medicinal Chemistry, University Hospital RWTH Aachen, D-52074 Aachen, Laboratory of Biochemistry, Institute for Interfacial Engineering, University of Stuttgart, D-70569 Stuttgart, §Physiological-Chemical Institute, University of Tübingen, D-72076 Tübingen, Germany, and ¶¶ To the Gaußius Laboratory, Toegespast Natuurwetenschappelijk Onderzoek-Prevention and Health, 2301 Leiden, The Netherlands

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1 The abbreviations used are: MIF, macrophage migration inhibitory factor; MIF-(50–65), sequence region 50–65 of human MIF; biotin-MIF-(50–65), analog of MIF-(50–65) with N-terminally linked biotin-macromolecule moiety; biotin-(C57S/C60S)-MIF-(50–65), analog of C57S/C60S-MIF-(50–65) with N-terminally linked biotin-macromolecule moiety; C57S/C60S-MIF-(50–65) or Ser-MIF-(50–65), bis-serine variant of MIF-(50–65) with Cys → Ser changes at positions 57 and 60; C60S-MIF, MIF mutant with a Cys → Ser mutation at residue 60; CALC, Cys-Ala-Leu-Cys motif; CXXC, Cys-Xaa-Xaa-Cys motif; CSN3, COP9 signalosome subunit 5; cyclo-MIF-(50–65), cyclo-57,60-[Asp57,Dap60]-MIF-(50–65); DTT, dithiothreitol; Fluo-MIF-(50–65), analog of MIF-(50–65) with N-terminally linked carboxyfluorescein moiety; Fluo-C57S/C60S-MIF-(50–65), analog of C57S/C60S-MIF-(50–65) with N-terminally linked biotin-macromolecule moiety; Grx, glutaredoxin; HEBD, bis-(2-hydroxyethyl)dithiothreitol; JAB1, c-Jun activation domain binding protein 1; LPS, lipopolysaccharide; LDL, protein-disulfide isomerase; rMIF, biologically active recombinant wild-type human MIF; TPOR, thiol-protein oxidoreductase; Trr, thioredoxin reductase; Trx, thioredoxin; HPLC, high pressure liquid chromatography; RP-HPLC, reverse phase HPLC; MPAK, mitogen-activated protein kinase; MALDI-TOF-MS, matrix-assisted laser desorption ionization/time-of-flight-mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; FCS, fetal calf serum; PBS, phosphate-buffered saline; ERK, extracellular signal-regulated kinase; VEGF, vascular endothelial cell growth factor; bFGF, basic fibroblast growth factor; DMEM, Dulbecco’s modified Eagle’s medium; DTT, dithiothreitol; MHC, major histocompatibility complex; CFSE, 5(6)-carboxyfluorescein di( N-hydroxysuccinimide ester); SPPS, solid phase synthetic protocols; DEX, dexamethasone; TNF, tumor necrosis factor; hMVEC, human microvascular endothelial cells; PDI, protein-disulfide isomerase; GGOR, glucocorticoid overriding; GIF, glycocytochrome inhibiting factor; Dap, diaminopropionic acid.
ity of protein kinases (13) and interacts with and regulates the activity of the transcriptional co-activator JAB1/CSN5 (14, 15). However, it is currently unclear what the upstream molecular events of these effects are. It has been considered that an observed catalytic thiol-protein oxidoreductase (TPOR) activity of MIF could be responsible, at least in part, for the cellular functions of MIF.

The TPOR family of proteins encompasses enzymes such as thioredoxin (Trx), glutaredoxin (Grx), protein-disulfide isomerase (PDI), or the disulfide bond proteins, with Trx being the prototype member of the family. TPORs catalyze the reduction of protein disulfides in a cysteine-based dithiol/disulfide-dependent process, and some family members also serve as potential protein-folding catalysts. TPOR proteins share a common Cys-Xaa-Xaa-Cys (CXXC) consensus motif within a homologous so-called Trx-fold, where the CXXC motif is located at the N terminus of an α-helix. MIF contains a CXXC TPOR consensus motif, with the residues Ala and Leu placed between the cysteines and also contains additional conserved residues that are frequently found N-terminally of the CXXC region (16). Although the overall three-dimensional structure of the MIF monomer shows a remote resemblance to the Trx monomer, MIF is not structurally homologous to the TPOR proteins, and the Cys-Ala-Leu-Cys (CALC) redox motif of MIF lies at the N terminus of a β-strand element with Cys57 located in the preceding loop (17, 18) rather than in a Trx-like fold. MIF exhibits TPOR activity in vitro being able to catalyze the reduction of both insulin and small molecular weight compound disulfides (19–21). Mutation of the CXXC cysteines of MIF results in partly or fully immunologically inactive MIF protein, an observation that has led to the suggestion that the TPOR activity of MIF is responsible in part for its immunological and cellular activities (14, 19–23). In addition, an involvement of MIF in the regulation of cellular redox processes is likely, as in addition to the above-mentioned findings, MIF was found to bind to and regulate the peroxiredoxin (24) and to inhibit oxidative stress-induced apoptosis (25). It has been speculated that the enzymatic activity of MIF could serve to bypass a potential receptor-mediated signaling pathway, to assist in MHC II antigen processing, or to mediate additional intracellular activities of MIF (6, 21, 26).

Thus, although MIF is not structurally related to the TPOR proteins, it shares with these proteins catalytic TPOR activity in vitro and the capability to participate in redox regulation in vivo. Moreover, MIF shares additional intriguing properties with Trx. Trx or adult T cell leukemia-derived factor plays a role in the regulation of cellular redox stress (27, 28) through its TPOR activity. Also, Trx, although originally discovered as a TPOR and ribonucleotide reductase enzyme, has recently been re-defined as a cytokine and immune mediator (29, 30).

The redox potential of the TPOR proteins is governed by both the overall three-dimensional structural constraints of the respective enzymes and the sequence composition of the CXXC motif together with the surrounding residues of the Trx fold (31–35). Short peptide fragments derived from TPORs that span the CXXC region and artificial mimetics thereof have therefore been investigated to correlate the sequence requirements with the redox potentials of the corresponding enzymes and to assess the influence of three-dimensional structural constraints (34–37). Of note, some of these small peptides were recently shown to function as catalysts of protein folding processes (35).

The three-dimensional structure of the CXXC region of MIF only has minor similarities to the CXXC/Trx-fold structure of the TPORs but MIF nevertheless exhibits TPOR-like activity, we surmised that during catalysis, the structure of MIF may be partially unfolded or changed. In this respect, it is noteworthy of mentioning that it has been suggested that a mono- or dimeric structure, possibly with slightly different conformational properties, but not the MIF trimer, may be the predominant MIF species under physiological conditions (38). Interestingly, thioredoxin reductase (Trr), a member of the TPOR family, also does not contain an apparent Trx fold structure but otherwise shares similar redox properties with the TPOR proteins, indicating that it may undergo a marked conformational change during catalysis (39). Computer-based secondary structure predictions had shown previously (19) that the CXXC sequence region of MIF has a high β-turn-forming propensity that is comparable with that of the TPOR enzymes. If altered conformational elements were involved in MIF-mediated redox catalysis and biological activity, it thus appeared possible that MIF-derived peptides featuring such properties could function as MIF mimetics.

Here we elected a 16-mer MIF peptide fragment spanning the CALC motif of MIF and encompassing the residues that are part of the predicted β-turn. The peptide ranged from residue Phe-50 to Ile-65 and was termed MIF-(50–65). It contained additional turn-stabilizing residues flanking the CXXC motif and covered the aromatic residue (Phe-50) which is part of extended CXXC sequence patterns according to Ellis et al. (16).

The peptide and its C57S/C60S bis-serine, biotinylated, and fluorescein-modified variants as well as a peptide analog covalently cyclized by a lactam bridge were synthesized by solid phase Fmoc chemistry, HPLC-purified, and investigated for their conformational, redox, and in vitro TPOR catalytic properties. Applying a variety of immunological and cellular assays that are characteristic of the immunological, inflammatory, and cellular functions of MIF in vivo, we then asked whether the 16-mer peptide may have retained MIF-like functional properties and could serve to mimic cellular MIF activities.

MATERIALS AND METHODS

Chemicals, Buffers, Recombinant MIF, and General Cell Culture Reagents—Protected amino acids were purchased from Rapp Polymer (Tübingen, Germany). All other peptide synthesis reagents were obtained from Bachem (Heidelberg, Germany). 5(6)-Carboxyfluorescein-N′-hydroxysuccinimide ester (CFSE) was from Roche Diagnostics. Analytical grade acetonitrile was from Mallinkrodt Chemical Works and from Merck. Trifluoroacetic acid, reduced and oxidized glutathione (GSH and GSSG), l-cysteine and l-cystine, 1,4-dithiothreitol (DTT), insulin, bis-(2-hydroxyethyl)-disulfide (HED), β-nicotinamide adenine dinucleotide phosphate (reduced form, NADPH), glutathione reductase, 1,3,4-dihydroxyphenylalanine methyl ester, sodium peroxide, lipopolysaccharide O111:B4 (LPS), and dexamethasone (DEX) were obtained from Sigma. All general cell culture reagents such as media, supplements, antibiotics, and serum (for the latter, see below) were from Invitrogen. NIH 3T3 and THP-1 cells were bought from the German Society for Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Cells were cultured by routine protocols at 37 °C in a humidified incubator with 5% CO2. Streptavidin-conjugated magnetic beads (Dynal) were purchased from Dynal Biotec ASA (Oslo, Norway). Streptavidin-coupled nanoparticles were a kind gift from Dr. G. Tovar (Fraunhofer IGB, Stuttgart, Germany). Biologically active recombinant human MIF (rMIF) was expressed, purified, and refolded as described previously (40). Vascular endothelial cell growth factor (VEGF) was purchased from REALLtech (Braunschweig, Germany), and basic fibroblast growth factor (bFGF) was bought from PeproTech (Rocky Hill, NJ). Crude endothelial cell growth factor was prepared from bovine hypothalamus as described by Maciag et al. (41).

1H]Thymidine was from Amersham Biosciences. Anti-phospho-ERK1/2 (E4, sc-7383) and anti-ERK1/2 (C16, sc-90) antibodies were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). Miscellaneous chemicals, solvents, and salts were from Sigma. All reagents were of the highest grade commercially available.

Synthesis and Purification of the MIF-derived Peptides MIF-(50–65) and C57S/C60S-MIF-(50–65) and Their Biotinylated, Fluoresceinated, Lactam-bridged Derivatives, and Control Peptides—Peptide synthesis...
was performed by solid phase synthetic protocols (SPPS) as described by us previously (42). The N terminus of the peptide was acetylated and the C terminus amidated. For synthesis of C57S/C60S-MIF-(50–65), serine residues were coupled at positions 57 and 60 instead of the active site cysteine. Biotinylation of MIF-(50–65) and C57S/C60S-MIF-(50–65) was performed at the N terminus of the fully protected and resin-bound peptides using normal coupling protocols in a mixture (2/1) of dimethylformamide and N-methyl-2-pyrrolidone, and an amino-caproate residue was also incorporated as a spacer between biotin and the peptide. N-terminal fluorescein-labeled forms of MIF-(50–65) and C57S/C60S-MIF-(50–65) were obtained by reaction of the side chain-protected, resin-bound peptides with CFSE which was applied in 1.7–2.3 molar excess. Reactions with CFSE were carried out for 3 h in a mildly basic (25% dimethylformamide and 25% N-methyl-2-pyrrolidone) medium. Synthesis and side chain-to-side chain cyclization of cyclo[57,60]Asp7,Dap60[MIF-(50–65)] and the linear control peptide [Asp7,Dap60][MIF-(50–65)] was performed according to a procedure published previously (42).

Briefly, as to the SPPS procedure in general, synthesis was performed on Rink resin applying the 9-fluorenlymethoxycarbonyl (Fmoc) group for temporary protection of the α-amino function. Side chains of trifunctional amino acids were protected with t-butyl (Ser), trityl (Cys), Trr (His), and t-butyl (Glu). Deprotection with simultaneous cleavage of the peptide from the resin was performed by treatment with 95% trifluoroacetic acid, 2.5% ethanedithiol, 2.5% water. Following evaporation of the cleavage mixture, trifluoroacetic acid under vacuum at 30 °C, 1% acetic acid was added to the resulting product, and the aqueous phase was extracted three times with diethyl ether. The aqueous phase was then lyophilized. The obtained crude product, which was obtained in its reduced form and in a purity of about 90%, was further purified by C18 reverse phase (RP) HPLC (250 × 6 mm; 100 Å pore size, 7 μm particle size; Grom, Herrenberg, Germany). Elution of the peptides was achieved with two different water/acetonitrile elution programs. The first program consisted of a gradient of 10% B to 90% B between 1 and 31 min; and the second elution program was 0–7 min at 30% B, followed by a gradient from 30 to 60% B for 30 min, with buffer A 0.058% trifluoroacetic acid, buffer B 90% acetonitrile, 0.05% trifluoroacetic acid, 0.01% acetic acid and water. The identity and purity of the peptides was verified by mass spectrometric analysis (see below and figure legends for details) and analytical HPLC. Following lyophilization, purified reduced peptide was stored at −20 °C until used further. Disulfide bridge-containing forms of the peptides were obtained by air oxidation in 0.1 M ammonium bicarbonate at a pH of 7.5. Following oxidation, the mixed disulfide species were collected on dry ice, deep frozen, and the C terminus amidated. For synthesis of C57S/C60S-MIF-(50–65), the oxidized peptide as well as the oxidized, and mixed disulfide species of the peptide were quantitated. The kinetic studies were performed at a ratio of 42/8 for both GSH/cysteine/cystine ratios of 42/8, 43/7, 44/6, 45/5, 46/4, 47/3, and 48/2 were applied. Afterward, 40 μl of 1 M H2PO4 was added to quench the reaction by reducing the pH to 2. The sample was immediately loaded on an analytical C18 RP-HPLC column (125 × 4 mm; 100 Å pore size, 5-μm particle size; Grom, Herrenberg, Germany). Elution was performed with a linear acetonitrile/water gradient (first gradient) as described above at a flow rate of 1 ml/min. Absorbance was monitored at 220 nm.

For the equilibrium and redox potential studies, the reactions were initiated by mixing equal volumes of 20 μg of MIP peptide fragment 50–65 and small molecular weight reductant. The final reaction volume was 100 μl. Oxidized MIF-(50–65) was generally equilibrated with a reference redox buffer containing varying ratios of GSH/GSSG or cysteine/cystine at a total concentration of 50 μM of redox cofactor. This corresponded to a large excess of the reference redox molecules over the peptide concentration (~400–500-fold for the reduced cofactor and 10–80-fold for the oxidized reference). Incubations were performed at 30 °C in 100 mM sodium phosphate buffer (pH 7.0) containing 1 mM EDTA for the indicated time intervals. Only in the preliminary scouting experiments, concentrations of the oxidized redox reference were in the concentration range of the peptide in some occasions. However, these experiments were not used for the quantification of the redox potential of MIF-(50–65). Reactions were stopped by adding 40 μl of 1 M H2PO4 to the 100 μl of the reaction mixture.

Glutathione ratios in the preliminary experiments were 50/10, 50/1, and 50/0.01. For the equilibrium studies and redox potential determinations with glutathione and cysteine, GSH/GSSG, and cysteine/cystine ratios of 42/8, 43/7, 44/6, 45/5, 46/4, 47/3, and 48/2 were applied. The kinetic studies were performed at a ratio of 42/8 for both GSH/GSSG and cysteine/cystine.

Following quenching with phosphoric acid, the samples were separated by C18 HPLC as above, and the peak areas of the reduced, oxidized, and mixed disulfide forms of the peptide were quantitated. In some experiments, peaks were collected and subjected to MALDI-TOF-MS (see above) to identify the oxidation state or mixed disulfide species.

For calculating the redox potential of MIF-(50–65), the equilibrium constant Kredox was determined according to the following Equation 1, with [Pox] and [Pred] representing the fraction of oxidized and reduced MIF-(50–65), respectively, as determined by the corresponding HPLC peak areas following the equilibrium incubations. [GSH] and [GSSG] and [Cys] and [cystine] represent the concentrations of the reduced and oxidized redox cofactors applied. The determination of Kredox through this equation is based on the assumption that each step in the multiple equilibria that occur in thiol/disulfide exchange reactions may be described by a two-state model (principle of detailed balancing (36, 44)). Thus, Kredox could be calculated although only one of the mono-mixed disulfide species could be identified and quantitated by HPLC in this study. Kredox values were calculated for both the glutathione and cysteine reference using all seven ratios applied. Kredox was calculated by two ways as follows: (a) the seven Kredox values were applied to calculate "Kredox mean," with the above equation was transformed into a linear regression type of equation y = m × x and Kredox determined from the m value. For both ways, Kredox was then used to calculate the redox potential E′c according to the Nernst Equation 2:

\[ E'_c = E'_0 - \frac{RT}{2F} \ln \left( \frac{[Pox]}{[Pred]} \right) \]  

(2)
with E'-(R-SH-R-SS-R) representing the standard redox potential for GSH/GSSG or cysteine/cysteine. Values of ~0.205 and ~0.225 V were assumed according to Szajewski and Whitesides (45) and Millis et al. (46), respectively.

The validity of the approach of assuming a two-state model was confirmed by the fact that the obtained values for E' - E'0 were nearly identical for the glutathione and cysteine redox references, because, when using the cysteine/cysteine pair, no significant fractions representing mixed disulfides were obtained (see "Results").

Enzymatic Assays—The catalytic activity of MIF-(50-65) was evaluated by two different enzymatic assays. The redox activity was addressed using the insulin reduction assay following a protocol described previously (19, 47). MIF-(50-65) was added to the assay from a 1 mM stock solution in 20 mM NaPP and tested at a final concentration of 40 M.

Tautomerase activity was measured using the n-dopachrome tautomerase test as described previously (48). Briefly, a fresh solution of n-dopachrome methyl ester was prepared by mixing 4 mM 1,3,4-dihydroxyphenylalanine methyl ester with 8 mM sodium phosphate for 5 min at room temperature and then placed directly on ice before use. Activity was then determined at room temperature by adding 100 M of dopachrome methyl ester to a cuvette containing 800 M of 25 mM potassium phosphate buffer (pH 6.0) and 0.5 mM EDTA. After 30 s, 100 nM MIF or 3.3 M MIF peptide was added to the cuvette, and the resulting decrease in absorbance at 475 nm was followed for 4 min using a Specol spectrophotometer (Jena Analytics, Jena, Germany).

Glucocorticoid Overriding Assay—Glucocorticoid counter-regulating activity of MIF, MIF-(50-65), and its bis-serine variant was assayed using a modification of the glucocorticoid overriding test described previously (3). Briefly, overriding by MIF and the peptides of the anti-inflammatory effect of DEX was analyzed through measuring LPS-stimulated tumor necrosis factor (TNF) production by THP-1 cells. Cells were taken from an early passage and were used no later than 3 weeks after having reached the exponential growth phase. THP-1 cells were used for these experiments because they are refractory to the bivalent cooperative overriding of MIF. Cells were treated with increasing concentrations of MIF or DEX for 1 h. Dexamethasone (Dex) was used as a positive control. The enzyme-linked immunosorbent assay (ELISA) was used to detect the TNF level in the cell supernatant.

Enzyme Reaction Monitoring—The TNF production was monitored by measuring the decrease in absorbance at 405 nm using a Specol spectrophotometer (Jena Analytics, Jena, Germany). The activity was calculated as the percentage of TNF release inhibited by the MIF or the peptides compared with the activity of DEX (100% inhibition).

Endothelial Cell Proliferation Assay—Human foreskin microvascular endothelial cells (hMVEC) were characterized as described previously (49, 50). Isolated cells were cultured on gelatin-coated 6-cm tissue culture plates in DMEM supplemented with 10% FCS and antibiotics (100 units/ml pen/strep). Then the cells were detached by trypsin/EDTA solution, allowed to adhere, and spread at a density of 104 cells per cm2 on the coverslips in 3.5-cm tissue culture plates and cultured overnight.

Endocytosis Studies of the Peptides

Induction of Phosphorylated ERK1/2—1 × 106 NIH 3T3 cells (passage 10-15) were plated in a 10-cm cell culture plate and incubated in DMEM containing 10% FCS for 24 h. The medium was changed and cells were incubated containing 0.5% FCS for 24-48 h. Then rMIF (MIF-(50-65), bis-serine-MIF-(50-65), bimbi-MIF-(50-65), bimbi-bis- serine-MIF-(50-65), and cyclo-MIF-(50-65) were reconstituted in NaPP buffer at a concentration of 530 M, and peptide solutions were added to the cells at a final concentration of 50 nM, 300 nM, 1 M, and 3.3 M. Controls were incubated with NaPP alone (volume according to the highest peptide concentration). Some plates were treated with 8 nM rMIF. Incubations were stopped at the indicated times (0-30 min) by washing the cells twice with cold PBS. Cells were then lysed with RIPA buffer (PBS (pH 7.4) containing 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1× proteinase inhibitor mixture I (Roche Diagnostics, Mannheim, Germany), and 1 mM sodium orthovanadate) and cellular proteins fractionated in 13.5% SDS-PAGE or 4-12% NuPAGE gels. Following transfer to nitrocellulose filter, Western blots were performed essentially as described previously (14), probed with anti-phospho-ERK1/2 antibody, and developed with the Super Signal West Dura Extended Duration ECL reagent (Pierce/KMP Laborchemie, St. Augustin, Germany). For counterstaining for ERK1/2, the membranes were stripped with 0.5% Tween 20 in TBS (20 mM Tris-HCl (pH 8), 150 mM NaCl) for 30 min and then reprobed with anti-ERK antibody.

Biolin-MIF-(50-65)-JAB1 Pull-down Assay—Pull-down experiments were performed essentially as described previously (14) except that bacterial lysates were used for the in vitro transcription/translation reactions. Briefly, 82-labeled JAB1 protein was generated using bacterial lysates (RINA GmbH, Berlin, Germany). Biolin-MIF-(50-65) and the biotinylated bis-serine variant were added to the lysates. Mixtures were incubated in low stringency buffer, and complexes precipitated with streptavadin-conjugated magnetic beads (streptavadin Dynabeads, Dynal Inc., Oslo, Norway). Beads were washed and boiled in Laemmli sample buffer and proteins run in a 13.5% SDS-PAGE gel. Detection of bound radiolabeled JAB1 was achieved by drying the gels, incubating them with AmplifyTM (Amersham Biosciences) for 30 min, and exposing to autoradiographic film.

Measuring the Effect of MIF and the Peptides on the Levels of p27Kip1—To determine the effect of MIF and the peptides on the level of p27Kip1, NIH 3T3 fibroblasts were serum-starved and synchronized as described before (14). Briefly, 0.25 × 105 NIH 3T3 cells were plated in 6-cm tissue culture plates in DMEM supplemented with 10% FCS and antibiotics (100 units/ml pen/strep) and 2 mM l-glutamine. After 6 h, the cells were washed with DMEM and incubated overnight in DMEM supplemented with 1% FCS, antibiotics, and 10% FCS. Then the cells were treated with different concentrations of rMIF or the peptides for 24 h at a density of 0.25 × 105 cells per 6-cm culture plate. During this period, the cells remained in the proliferating phase. The cells were harvested, and lysis was performed in RIPA buffer and collected by centrifugation. The supernatants were mixed with 2× Laemmli buffer, boiled, and applied to SDS-PAGE (13.5%) and blotted to a nitrocellulose membrane as described previously (14). Gels and Western blots on the concentration dependences were performed with the NuPAGE (4-12%) system as described (25). Equal loading was adjusted for following Bradford protein assay of the cell lysates (52). A mouse anti-p27 monoclonal IgG (F-8, dilution 1:1000; Santa Cruz Biotechnology or anti-p27 antibody, Transduction Laboratories, Lexington, KY) in combination with an anti-mouse peroxidase-coupled antibody was used to detect p27Kip1. Bands were visualized using ECL reagent as above.

Endocytosis Studies of the Peptides—0.2 × 106 NIH 3T3 cells in DMEM supplemented with 10% FCS and antibiotics (100 units/ml pen/strep) and 2 mM l-glutamine were plated on poly-l-lysine-coated coverslips in 3.5-cm tissue culture plates and cultured overnight. Cells were washed 1× with PBS and DMEM with 0.5% FCS was added. The fluorescently labeled peptides were added at a concentration of 10 M and incubated with the cells for 60 min. Afterward, cells were washed twice with acid glycine buffer (150 mM NaCl, 50 mM glycine (pH 3)) and twice with PBS. Then cells were fixed for 30 min in 4% paraformaldehyde at 37 °C and washed with PBS for another three times. Coverslips were inverted, Fluoromount added, and dried overnight. Fluorescently labeled rMIF, labeled with carboxyfluorescein using a kit from Roche Diagnostics as described previously (14), was used for comparison and to establish the endocytosis assay in the fibroblast cell line. Cell-bound fluorescence was scored by fluorescence microscopy and quantitated with the Soft Imaging Systems software (Munster, Germany).

RESULTS

Design, Synthesis, Purification, and Chemical Properties of MIF-(50-65) and Its Derivatives—Small peptide derivatives of TPORs containing as few as 6 residues have been reported to retain some of the redox and biochemical properties of their parent protein molecules (35, 36). Election of peptide fragment 50-65 of the cytokine MIF (the nomenclature refers to the
cDNA sequence of human MIF with the Met-1 residue constituting amino acid number 1 of the MIF sequence and does not take into account that Met-1 is post-translationally processed) as a potentially minimized MIF derivative was based on peptide sequence MIF-(56-65), which had been shown previously to exhibit low but measurable MIF-like catalytic oxidoreductase activity (19). MIF-(50-65) also spanned the CXXC motif of human MIF and encompassed the β4-strand. Other than MIF-(56-65), which covered the β4-strand only, MIF-(50-65) was chosen to include additional residues that could serve to stabilize secondary structure interactions. In fact, it has been proposed for the TPORs that formation of a stable β-turn structure in the CXXC region is associated with oxidoreductase activity (37). Therefore, MIF-(56-65) was N-terminally extended up to residue Phe-50 to enable formation of additional intramolecular CXXC structure-stabilizing interactions. At the same time, this meant that MIF-(50-65) encompassed the extended TPOR motif according to Ellis et al. (16), which included the Phe residue at position 50. Initial secondary structure predictions by the methods of Chou and Fasman (53) and Garnier et al. (54) indicated that sequence 50–65 had a reasonably high β-turn-forming probability of up to 37.5% and was sufficiently long to form a stable intramolecular β-sheet structure (55). Downstream of the CXXC motif, the sequence covered the residues up to position 65 to contain two Ser residues and His-63. These residues as well as the two serines lying upstream of CXXC were deemed potentially important due to their principal capability of stabilizing the cysteine thiolate or generating a dipole function (56). Also, MIF-(50-65) contained an inverse CXXS motif (corresponding to residues 57 → 54). CXXS groups have been recognized recently as fold independent redox motifs that are indicative of TPORs (57).

As a potentially inactive derivative of MIF-(50-65), C57S/C60S-bis-serine-MIF-(50-65) was chosen, whereas biotinylated MIF-(50-65) and biotin-C57S/C60S-MIF-(50-65), which were devised for binding assays, were designed to carry N-terminal biotinylation with an aminocaproate group as a spacer to minimally interfere with the conformation of the peptide. For cell uptake studies, fluorescent peptides were synthesized with a carboxyfluorescein moiety linked to the N terminal. To probe further the conformational changes of MIF-(50-65) that were expected to occur upon intramolecular disulfide formation, the covalently cyclized analog of MIF-(50-65), cyclo[Asp57,Dap60]MIF-(50-65) (abbreviated as cyclo-MIF-(50-65)) and its corresponding linear control peptide [Asp57,Dap60]MIF-(50-65) were applied. The sequences of the peptides are shown in Fig. 1A.

SPPS gave the peptides in good yield. Crude products of the syntheses except for that of the fluoresceinated bis-serine variant consisted of the desired product to a degree more than 90%. The crude product of Fluo-C57S/C60S-MIF-(50-65) contained a significant fraction of a synthesis side product that was removed by an additional interim RP-HPLC step. Together, using RP-HPLC purification, all peptides were obtained in their reduced forms at purities greater than 98% (Fig. 1, B–E). The obtained M + 1 masses of the products were in good agreement with the theoretical mass values (measured mass of MIF-(50−65) = 1648.9 Da; expected: 1648.9 Da; measured mass of C57S/C60S-bis-serine-MIF-(50−65) = 1617.0 Da, theoretical: 1617.8 Da; measured mass of biotin-Aca-MIF-(50−65) = 1945.8 Da, expected: 1945.5 Da; measured mass of biotin-Aca-C57S/C60S-MIF-(50−65) = 1914.6 Da, expected: 1915.2 Da; measured mass of Fluo-MIF-(50−65) = 1964.4 Da, expected: 1964.1 Da; Fluo-C57S/C60S-MIF-(50−65) = 1934.8 Da, expected: 1934.0 Da; measured mass of cyclo[Asp57,Dap60]MIF-(50−65) = 1625.4 Da; expected: 1626.8 Da; measured mass of [Asp57,Dap60]MIF-(50−65) = 1643.3 Da; expected: 1643.8 Da).

Incubation of MIF-(50−65) with an excess of DTT led to a rapid and complete reduction of MIF-(50−65). Fig. 1F shows the HPLC elution profile of oxidized versus reduced MIF-(50-65) with the reduced species eluting 0.7 min earlier than the oxidized peptide. The HPLC procedure and elution profile was highly reproducible and was thus also used for the generation of the reduced and oxidized species of MIF-(50-65) at a preparative scale. Under these reducing conditions, no intermediates were observed. Mass spectrometric analysis using either liquid chromatography-coupled electrospray MS or MALDI-TOF-MS showed the expected mass difference of 2 Da between the species, with oxidized MIF-(50−65) having a M + 1 mass of 1646.7 Da (theoretical M + 1 mass = 1646.9 Da) and reduced MIF-(50−65) having a M + 1 mass of 1649.0 Da (theoretical M + 1 mass = 1648.9 Da). Of note, the oxidized and reduced species of full-length MIF eluted in the same order from the C18 HPLC column (reduced rMIF, 17.56 ± 0.44 min, n = 5; oxidized rMIF, 17.85 ± 0.34 min, n = 5). On the other hand, the shorter MIF fragment, MIF-(56−65) showed an inverse elution profile, with the oxidized species eluting 0.6 min before the reduced one. Thus, although the differences in elution time observed for the full-length MIF species were not significant (p > 0.05), this indicated that MIF-(50−65) was similar to rMIF with respect to the conformational changes and subsequent changes in molecular surface hydrophobicity that occur during oxidation/reduction.

Disulfide-dependent β-Turn Formation of MIF-(50−65)—CD spectropolarimetry was applied to estimate the conformational properties of MIF-(50−65). Near-UV CD analysis confirmed intramolecular disulfide formation of MIF-(50−65) following air oxidation of the peptide. Oxidized MIF-(50−65) showed a CD signal between 270 and 285 nm that is characteristic of a disulfide group and that was not detectable when the reduced peptide or the bis-serine derivative were analyzed by near-UV CD (Fig. 2A). Oxidation-induced disulfide formation was reversible. When oxidized MIF-(50−65) was incubated with DTT for a period from 10 min to 24 h, the CD signal at 270–285 nm decreased successively (Fig. 2B). After 24 h of reduction, the spectrum was superimposable to that of fully reduced MIF-(50−65). The kinetics of the reduction process indicated that the peptide can readily undergo oxidation/reduction processes.

The far-UV CD spectra of reduced MIF-(50−65) and C57S/C60S-MIF-(50−65) exhibited strong minima at about 200 nm which indicated that both peptides were predominantly unordered, with C57S/C60S-MIF-(50−65) being significantly more unordered than MIF-(50−65). Disulfide formation of MIF-(50−65) was accompanied by a clear increase in β-turn structure. Fig. 2C shows that the far-UV CD spectrum of oxidized MIF-(50−65) exhibited a well pronounced minimum at 225–230 nm (58). By contrast, reduced MIF-(50−65) and the bis-serine mutant did not exhibit significant minima in this wavelength range. This indicated that formation of the disulfide bridge in MIF-(50−65) was associated with β-turn stabilization. The magnitude of the random coil minimum and thus of random coil content was significantly reduced in oxidized MIF-(50−65) as compared with reduced MIF-(50−65) which indicated that β-turn stabilization was associated with an increase in conformational ordering of this molecule. Of note, disulfide formation-induced β-turn stabilization of MIF-(50−65) was further confirmed by CD analysis of cyclo-MIF-(50−65). This peptide analog of MIF-(50−65) contained a covalent side chain-to-side chain lactam bridge between residues 57 and 60 which had been substituted by Asp and Dap, respectively. The ring struc-
ture in this cyclic analog had the same size as the ring structure that was formed in the disulfide-bridged oxidized form of MIF-(50–65). The CD spectrum of this analog exhibited a pronounced minimum at 225 nm that was even stronger than that for oxidized MIF-(50–65), indicating that the cyclic structure resulted in significant -turn stabilization (Fig. 2D). By contrast, the corresponding linear control peptide [Asp57,Dap60]MIF-(50–65) had no minimum at this wavelength but was rather predominant in a random coil conformation as evidenced by a marked minimum at 200 nm (Fig. 2D). Thus, the far-UV CD spectrum of cyclo57,60-[Asp57,Dap60]MIF-(50–65) was similar to that of oxidized MIF-(50–65), whereas the spectrum of [Asp57,Dap60]MIF-(50–65) was most similar to that of C57S/C60S-MIF-(50–65).

**Thiol/Disulfide Equilibrium Measurements, Mixed Disulfide Formation, and Redox Potential of MIF-(50–65)**—We next were interested in investigating the redox properties of MIF-(50–65) in more detail. This encompassed equilibrium measurements and determination of the redox potential of the peptide. Redox equilibrium measurements were performed with both glutathione (GSH/GSSG) and cysteine/cystine as reference redox pairs. Both references were applied in excess over MIF-(50–65). Reactions were quenched, and the distribution of...
the peptide species was analyzed by quantitative RP-HPLC and, in some experiments, by mass spectrometric analysis.

Preliminary experiments were performed with GSH/GSSG, using ratios of 50/10, 50/1, and 50/0.001. At a ratio of 50/1, most of the oxidized peptide (elution time, 14.9 min) was converted into the reduced form (14.2 min) within 2 h of reduction. However, this analysis also indicated that several peaks with shorter elution times appeared. By using a ratio of 50/0.001, essentially the entire amount of oxidized peptide was reduced within 2 h with no additional peaks observed. By using a ratio of 50/10, only a small portion of the oxidized peptide was reduced in 2 h, and also after 48 h, additional strong peaks were seen, indicating that stable mixed disulfides may have formed (data not shown).

Equilibrium and redox potential measurements were then performed at a fixed total concentration of GSH/GSSG or cysteine/cystine of 50 mM. Mixed disulfide formation was measured at a ratio of 42/8, incubating the mixtures for 20 h. Under these conditions, a similar chromatogram containing additional peaks indicative of mixed disulfide species was obtained as for the 50/10 ratio in the scouting experiments (Fig. 3A).

Kinetic studies (Fig. 3B) showed on the one hand that equilibrium was reached at 20 h, as no changes in the chromatogram were observed toward 42 h and, on the other hand, indicated that a mixed disulfide species peak at an elution time of 13.4 min appeared as early as 10 min after starting the reaction. Another potential mixed disulfide peak was observed at 30 min and had an elution time of 12.4 min. Both peak intensities as well as that of oxidized peptide declined over time as the peak of reduced MIF-(50–65) increased.

The identity of the potential mixed disulfide species was studied by MALDI-TOF-MS of the collected peak fractions at equilibrium conditions. Table I summarizes the obtained masses and their assignments. The equilibrium mixture contained a significant portion of mono-mixed disulfide (GS-S-MIF-(50–65)) (~30%) and ~10% of the di-mixed disulfide species (GS-S-MIF-(50–65)-S-SG). Less than 2% MIF-(50–65) dimer was measured, confirming preliminary prior data that covalent dimerization is not favored for either MIF or MIF-derived CXXC-spanning peptides. Only one mono-mixed disulfide species could be detected. It is unclear whether only one mono-mixed species formed, whether one species was unstable,
FIG. 3. Thiol/disulfide equilibrium studies of MIF-(50–65) with the GSH/GSSG redox reference pair and evidence for stable mixed disulfide formation. Reactions were analyzed by analytical C18 HPLC. A, equilibrium mixtures (20 h) of purified oxidized MIF-(50–65) with a molar excess of GSH/GSSG at ratio of 42/8 mM indicated that a stable mono-mixed disulfide and stable di-mixed disulfides had formed and that reduced MIF-(50–65) was the predominant species at equilibrium. The second mono-mixed disulfide species could not be detected.

B, kinetics of the reduction and formation of mixed disulfides in mixtures of oxidized MIF-(50–65) with GSH/GSSG (42/8 mM). Reactions from 0 min to 42 h were quenched with 1 M H₃PO₄, and chromatograms were recorded as described under "Materials and Methods." C summarizes the chromatograms of B. A mono-mixed disulfide species formed early on and was prominent at 10 min, indicating that it was a transition state for the formation of reduced MIF-(50–65). Detectable from 30 min on, an apparently stable di-mixed disulfide species formed. The relative reduction of the percentage of mono-mixed disulfide species between 30 min and 5 h indicated that this species was a transition state for reduced MIF-(50–65). Changes in absorbance between 20 and 42 h were not significant; equilibrium was reached at around 20 h. Chromatograms represent the elution times of the peptide species plotted over the absorbance at 220 nm.
or whether the elution times of both species were very similar such that the peaks could not be resolved. Fig. 3C summarizes the kinetics of the reduction reaction.

Initial measurements with cysteine/cystine at a ratio of 42:8 indicated that the reduction process was much faster than when the GSH/GSSG redox pair was used. After 10 min, reduced MIF-(50–65) represented already ~60% of total peptide. For comparison, at this time point only <5% of peptide were in the fully reduced state when the glutathione mixture was applied (see Fig. 3B). Equilibrium was reached already at 5 h (Fig. 4A). A detailed kinetic analysis of the cysteine-dependent reduction between 0 and 10 min then demonstrated that a marked portion of reduced MIF-(50–65) was already detectable at 2 min (~30%). By 10 min, reduced MIF-(50–65) was the predominant species present (see above). Mixed disulfide formation was fast and transient, with only small peaks (~5% each) with elution times of 13.5 and 12.5 min observed at 2 min, which further declined in intensity toward 10 min (Fig. 4B).

The redox potential \( E'_o \) of MIF-(50–65) was determined at equilibrium using various ratios of GSH/GSSG and cysteine/ cystine. The detectable peptide species were quantitated by HPLC and the relative abundance of the peak areas used to determine \( K_{mi} \), which in turn was used to calculate \( E'_o \) through the Nernst equation (see “Materials and Methods”). By using GSH/GSSG as a reference redox pair, a \( E'_o \) value of ~0.255 V was obtained. Applying cysteine/cystine, \( E'_o \) was found to have a value of ~0.255 V. The latter result confirmed that the calculation of \( K_{mi} \) can be based on the two-state model. Together, these measurements showed that the redox potential of MIF-(50–65) was a strongly negative one.

**MIF-(50–65) Has Catalytic Oxidoreductase Activity in Vitro**—MIF was shown to exhibit thiol-protein oxidoreductase activity in the insulin reduction and HED transhydrogenase assay (19). Here we demonstrated that MIF-(50–65), at a 10-fold higher concentration than full-length rMIF, was able to catalyze insulin reduction. This activity was dependent on the presence of the CXXC cysteine residues as the bis-serine mutant peptide was inactive (Fig. 5A). MIF-(50–65) also catalyzed the reduction of 2-HED, although this activity was very low (only 17% of rMIF at a 10-fold higher concentration) and not significant (data not shown). As expected from previous structure activity relationship studies on the oxidoreductase and tautomerase activities of MIF (23), MIF-(50–65) did not catalyze the conversion of D-dopachrome methyl ester, whereas full-length rMIF potently catalyzed this reaction (Fig. 5B).

Thus, these findings confirmed the notion that the TPOR activity of MIF is mediated by a sequence stretch around the CXXC motif. As for other TPORs, MIF-derived CXXC-spanning peptide fragments appear to bear some of the catalytic TPOR activity of the full-length protein. The result also confirmed previous notions that the TPOR and tautomerase activities of MIF are mediated by distinct regions of the MIF molecule (23, 59).

**MIF-(50–65) Exhibits MIF-like Immunostimulatory Activity**—The immunostimulatory activity of MIF has been best characterized by the capacity of MIF to override the antiinflammatory and immunosuppressive activity of glucocorticoids (3). The glucocorticoid antagonistic activity of MIF is unique among cytokines and has been shown to be relevant in vivo (3, 60). A CXXC mutant of MIF was found to have significantly reduced glucocorticoid overriding (GCOR) activity compared with the wild-type protein, indicating that the TPOR activity could be important for GCOR. We therefore hypothesized that, in addition to exhibiting oxidoreductase activity, MIF-(50–65) may have MIF-like GCOR activity. When testing overriding of the suppression by DEX of TNF secretion from LPS-stimulated THP-1 monocytes, MIF-(50–65) was in fact found to have GCOR activity. At a concentration of 3.3 \( \mu M \), MIF-(50–65) led to an overriding of ~45%. Lower concentrations of 1 \( \mu M \) and 50 nM had no activity. The activity of 3.3 \( \mu M \) MIF-(50–65) was comparable with that obtained for 0.16 \( \mu M \) rMIF (Fig. 6A). The bis-serine variant did not show any GCOR activity at any of the concentrations tested (50 nM to 3.3 \( \mu M \)). Thus, although the concentration of the peptide needed was much higher than for full-length MIF, we concluded that the 16-mer MIF peptide had at least partial MIF-like immunologic activity.

Protein minimization has been attempted for several cytokines and hormones and has only been successful in some cases (see “Discussion”). Thus, to confirm whether MIF-(50–65) exhibited an immuno-stimulatory potential characteristic of MIF, we tested cellular activation by the peptide in several other experimental settings. MIF has been shown to modulate the proliferation of a number of different cell types. For example, a potent proliferative activity of MIF has been described in fibroblasts (13). We found that MIF modulated the proliferation of primary vascular endothelial cells. Proliferation-modulating effects of MIF were observed for both human microvascular endothelial cells (MVEC) and human umbilical vein endothelial cells (latter not shown). Regulation of the cellular activation state of endothelial cells by MIF could be important for MIF-mediated inflammatory reactions in human atherosclerosis (15). We show that rMIF enhanced the proliferation-inducing effect of bFGF and VEGF on MVEC in a concentration-dependent manner (Fig. 6, B and C). Significant induction of proliferation by MIF was observed for MIF concentrations ranging from 8 to 400 nM. Importantly, the activity measured was based on the use of an assay with primary cells rather than immortal cell lines. It was therefore tested whether MIF-(50–65) exhibited MIF-like activity in this assay. We found that MIF-(50–65), like MIF, enhanced the inducing effects of both bFGF and VEGF on MVEC proliferation in a concentration-dependent manner. Significant proliferation-stimulating activity was observed at concentrations over 1 \( \mu M \). The bis-serine variant of MIF-(50–65) had no significant activity (Fig. 6, B and C).

**MIF-(50–65) Modulates Intracellular Signaling Targets of MIF**—We next asked whether the 16-mer peptide analog was also capable of modulating intracellular pathways in a MIF-

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**Table I**

| Species | Relative abundance at equilibrium | Retention time | Expected M + Na⁺ mass for species | Experimentally determined M + Na⁺ mass |
|---------|----------------------------------|----------------|----------------------------------|--------------------------------------|
| oxMIF (50–65) | 18 | 14.9 | 1688 | 1669.0 |
| redMIF (50–65) | 40 | 14.4 | 1670 | 1670.7 |
| mono-mixed disulfide (GS-S-MIF (50–65)) | 30 | 13.5 | 1976 | 1976.5 |
| DI-mixed disulfide (GS-S-MIF (50–65)-S-SG) | 10 | 12.4 | 2281.6 | 2281.3 |
| Dimer | <2 | 6 | 3292 Da | 3295.1 |

\(^a\) \( M + H⁺ \) mass; equilibrium mixtures were quenched with phosphoric acid and fractionated by C18 HPLC as described under “Materials and Methods.” Peaks of the indicated elution times were collected, immediately frozen on dry ice, lyophilized, and analyzed by MALDI-TOF-mass spectrometry. Only one mono-mixed disulfide species could be detected.
like manner. As activation of the ERK1/2 kinase had been linked to the proliferation-stimulating effect of MIF (13), we first investigated the effect of the peptide on the formation of intracellular endogenous phospho-ERK1/2 levels in NIH 3T3 fibroblasts. In initial experiments, we confirmed the reported stimulatory effect of rMIF on ERK1/2 activity in these fibroblasts (Fig. 7A, right panel). We then compared the effect of MIF with the potential effect of MIF-(50–65). Because we wanted to later on probe whether MIF-(50–65) could bind to the cellular binding partner of MIF, JAB1, and biotinylated MIF-(50–65) was to be used for the binding studies, we applied both biotinylated MIF-(50–65) and MIF-(50–65) in the ERK1/2 phosphorylation experiments and included the corresponding bis-serine MIF-(50–65) peptides for comparison. Both biotin-MIF-(50–65) (Fig. 7A, top panel) and MIF-(50–65) (Fig. 7B, middle panel) stimulated the phosphorylation of ERK1/2. Induction of the phosphorylation of ERK1/2 by the peptide was concentration-dependent, with the maximal induction observed at 330 nM and with some effect already observed at concentrations as low as 3 nM peptide. The activity of MIF-

Fig. 4. Thiol/disulfide equilibrium studies of MIF-(50–65) with the cysteine/cystine redox reference pair. Mixed disulfide formation occurred immediately after mixing the components and appeared to be transient only. Reactions were analyzed by analytical C18 HPLC. A, time course of thiol/disulfide exchange reactions (cysteine/cystine ratio of 42/8 mM) from 0–20 h. No change in elution profile was observed after 5 h. B, time course between 0 and 8 min. Peak representing substantial amounts of mixed disulfide species were not observed even at 2 min. Chromatograms were recorded as described under “Materials and Methods” and represent the elution times of the peptide species plotted over the absorbance at 220 nm.
MIF-(50–65) Exhibits MIF-like Activities

Fig. 5. MIF-(50–65) exhibits catalytic thiol-protein oxidoreductase activity. A, MIF-(50–65) but not C57S/C60S-MIF-(50–65) reduces insulin disulfides. The catalytic activity was measured in a turbidometric assay that relied on the insolubilization of the insulin B-chain following disulfide reduction using glutathione as a cofactor as described under “Materials and Methods.” The absorbance at 650 nm representing light scattering by B-chain aggregates is plotted over the reaction time. B, MIF-(50–65) does not share with full-length human MIF the ability to catalyze the conversion of β-dopachrome methyl ester. The plot shows the absorbance at 475 nm versus the reaction. The assay is described in detail under “Materials and Methods.”

(50–65) was ~1000-fold higher than that of the bis-serine control peptide, as this peptide exhibited only minor activity at 3.3 μM (Fig. 7A, middle panel). Thus, MIF-(50–65) was able to stimulate specifically the ERK1/2 MAPK pathway that is activated by MIF in fibroblasts. A first indication as to the mechanism of ERK1/2 activation was obtained by testing the cyclo-MIF-(50–65) in comparison with a linear control peptide. The analysis showed (Fig. 7A, lower panel) that this non-cysteine-containing, β-turn-stabilized MIF-(50–65) analog stimulated ERK phosphorylation comparable with MIF-(50–65), confirming the importance of the presence of a β-turn conformation for triggering this signal transduction process (see “Discussion” for an initial mechanistic model).

JAB1/C5N5 has been shown to be an intracellular binding partner of MIF. MIF can modulate intracellular signaling pathways through JAB1, which has been one of the few characterized signaling targets of MIF (14). It was therefore of interest if the MIF-derived peptide was able to bind directly to JAB1. Biotinylated MIF-(50–65) was applied to probe for complex formation between radioactively labeled JAB1 and the peptide by pull-down assay. Fig. 7B shows that MIF-(50–65) specifically bound to JAB1. This finding was thus in line with previous observations that MIF-(50–65) could compete with MIF for JAB1 binding (14). Complex formation between biotin-MIF-(50–65) and JAB1 was further confirmed by pull-down of complexes of biotin-MIF-(50–65) and the so-called MPN domain of JAB1. In this setting, complexes of biotin-MIF-(50–65) and in vitro translated MPN protein were precipitated by newly developed streptavidin-conjugated nanoparticles and MPN directly measured by MALDI-TOP-MS from surface-immobilized streptavidin nanoparticle (data not shown; to be published in detail elsewhere). Interestingly, the bis-serine derivative of MIF-(50–65) also bound to JAB1. This finding was also in agreement with prior competition experiments that had shown that the serine variant also interfered with MIF binding to JAB1. Together, these data indicated that the sequence stretch 50–65 was sufficient for the binding of MIF to JAB1. They also indicated that the CXXC cysteine residues were dispensable for binding.

Because in the cellular activation assays (GCOR, proliferation, and ERK1/2 kinase assay), the bis-serine variant of the peptide was essentially inactive, we wished to compare the effects of the two peptides with respect to cellular targets/activities which MIF can regulate in a JAB1-dependent manner. As MIF had been demonstrated to stabilize p27\(^{G1}\) levels in a JAB1-mediated way, we studied the effect of MIF-(50–65) on cellular p27 levels and compared the effect to that of full-length rMIF and C57S/C60S-MIF-(50–65). Fig. 7C shows that MIF-(50–65) had a comparable p27 concentration-enhancing effect as rMIF. The effect was concentration-dependent with a maximum induction measured at peptide concentrations of 2–3 μM. As seen in the JAB1 binding experiments, the bis-serine variant exhibited a similar activity. In conjunction with the result of the binding study, this indicated that sequence stretch 50–65 is important and sufficient for JAB1 binding and modulation of at least some of the JAB1 activities by MIF. By contrast for the GCOR, cell proliferation-stimulating and MAPK activity assays, the presence of the CXXC cysteine residues and/or β-turn conformation appeared to be critical.

MIF-(50–65) and the Bis-serine Peptide Are Endocytosed by MIF Target Cells at a Similar Rate—Binding to JAB1 and modulation of p27 levels by the peptides suggested that both MIF-(50–65) and the bis-serine analog can be taken up by MIF target cells to then act intracellularly. We thus confirmed that the peptides, like MIF, were endocytosed by NIH 3T3 cells. Fluorescently labeled MIF-(50–65) and C57S/C60S-MIF-(50–65) were both found to be endocytosed by the fibroblasts within 60 min (Fig. 8 and data not shown). However, uptake of the peptides was less efficient than that for fluorescently labeled rMIF (Fig. 8A), for which marked cell-bound fluorescence was already seen after 10 min (latter not shown). Because the bis-serine variant of MIF-(50–65) was essentially inactive in the GCOR, proliferation, and ERK assays, but exhibited MIF-like activity on the putative intracellular MIF signaling targets JAB1 and p27, it appeared important to compare directly the endocytosis efficiency of MIF-(50–65) and the bis-serine variant. Quantification of the cell-bound fluorescence for the two peptides showed that the two peptide species were endocytosed at a similar rate (Fig. 8B). This suggested that different activity profiles observed for the peptide species in the various activity assays were not due to differences in their cellular uptake rates. The higher uptake rate of rMIF as compared with the peptides may in part be due to the higher labeling grade of rMIF (14) but may also indicate that MIF but not the peptides could recruit additional proteins to facilitate uptake and signaling.

Together, the immunological and cellular activation assays
MIF-(50–65) Exhibits MIF-like Activities

MIF is a relatively small protein mediator, for which numerous biological activities have been demonstrated. Due to its potent inflammation- and immuno-regulatory properties, MIF has mostly been viewed as a cytokine, although endocrine-like functions have been described and although MIF differs from typical cytokines with respect to several characteristics (reviewed in Refs. 5 and 6). A membrane receptor that could serve to mediate the various activities of MIF has not yet been identified, whereas two intracellular proteins, the transcriptional co-activator JAB1 (14) and peroxiredoxin (24), have been shown to bind to MIF. Functional analysis indicated that JAB1 activity is antagonized by MIF (14). Nevertheless, as MIF triggers certain other typical signal transduction pathways such as the ERK1/2 MAPK pathway, it is likely that some of the functions of MIF are funneled through receptor-mediated pathways.

In addition, two distinct catalytic activities, i.e., a TPOR and a dopachrome isomerase activity, have been described for MIF (19, 48). These activities have clearly been demonstrated in in vitro studies, but their physiological role has remained open. Consisting of only 114 residues, it is not surprising that for MIF, no typical domain structure has been identified. However, previous structure activity studies have indicated that the catalytic activities of MIF reside within small sequence motifs (23, 61). The TPOR activity is mediated by a CXXC sequence in the middle of the MIF molecule, whereas the isomerase activity is dependent on an N-terminal Pro residue. In line with the latter finding, MIF-(50–65) did not show any d-dopachrome tautomerase activity. The TPOR activity is further enhanced by N-terminal residues (23), whereas for the tautomerase function, C-terminal residues and additional other residues critically participate in forming the substrate pocket (59, 62).

Our previous studies on N-terminally truncated MIF mutants indicated that for certain typical MIF activities the N-terminal region of the molecule is dispensable (23). However, these prior minimization attempts only led to MIF derivatives with truncations of less than 10 residues. Also, C-terminally

MIF-(50–65) has GCOR activity as demonstrated in a THP-1 monocyte-based overriding assay (see “Materials and Methods”). The activity of MIF-(50–65) and its bis-serine variant was compared with the activity of full-length human MIF. Lower concentrations of MIF-(50–65) did not exhibit any GCOR activity. Percent GCOR activity is plotted, whereby 100% overriding was defined as that amount of secreted TNF that was induced by LPS. Suppression of LPS-induced TNF by 10^{-8} M dexamethasone (DEX, Dex) represented 0% overriding activity. Asterisks indicate statistically significant values (p < 0.05) in comparison to the “DEX + LPS value” as determined by unpaired Student’s t test. Values represent the mean ± S.D. of 3–18 measurements. B and C, MIF-(50–65) but not C57S/C60S-MIF-(50–65) enhances the bFGF-induced (B) and VEGF-induced (C) proliferation of microvascular endothelial cells (MVECs) comparable with full-length human MIF. The effect of various peptide concentrations was compared with that of 400 nM full-length human MIF. Lower concentrations of MIF-(50–65) did not show any GCOR activity. Percent GCOR activity is plotted, whereby 100% overriding was defined as that amount of secreted TNF that was induced by LPS. Suppression of LPS-induced TNF by 10^{-8} M dexamethasone (DEX, Dex) represented 0% overriding activity. Asterisks indicate statistically significant values (p < 0.05) in comparison to the “DEX + LPS value” as determined by unpaired Student’s t test. Values represent the mean ± S.D. of 3–18 measurements. B and C, MIF-(50–65) but not C57S/C60S-MIF-(50–65) enhances the bFGF-induced (B) and VEGF-induced (C) proliferation of microvascular endothelial cells (MVECs) comparable with full-length human MIF. The effect of various peptide concentrations was compared with that of 400 nM full-length human MIF. Control cells were not treated at all (cells), incubated with growth factor alone (−), or incubated with the same volume of phosphate buffer as that used for the peptide incubations (NaPP). Asterisks indicate statistically significant bars (p < 0.05 according to the unpaired t test) with respect to the “cells + growth factor alone (−)” bars. Bars represent the mean ± S.D. of 3 measurements. Proliferation of MVECs is plotted as incorporated radiolabeled thymidine in dpm values.
MIF-(50–65) modulates signal transduction processes in a MIF-like fashion. A, concomitant with the growth-stimulatory effect of MIF-(50–65) as seen in Fig. 6, the peptide induces ERK1/2 kinase activity to enhance endogenous phospho-ERK1/2 levels in NIH 3T3 fibroblasts (upper panel) in an MIF-like fashion. Phospho-ERK1/2 levels were analyzed by Western blot with phospho-specific antibody, and ERK1/2 was analyzed for control. Cells were incubated for 0–30 min with the peptides and 0–120 min with full-length MIF (rMIF, latter 3 time points not shown) (upper panel), and peptides were tested over a wide concentration range from 0.33 nM to 3.3 μM (middle panel). Densitometric analysis of the blots following treatment of the cells with the peptides indicated that phosphorylation of ERK1/2 was stimulated about 2-fold (top and
truncated MIF variants, which retained some of the activities of MIF, consisted of over 100 residues (40, 59).

C-terminal mutants were found to be inactive in the GCOR assay (17, 38). The observation in our current study that MIF-(50–65), which naturally lacks the C terminus of MIF, exhibited GCOR activity indicates that different mechanisms could underlie the GCOR-stimulating activity of MIF and the peptide. On the other hand, previous GCOR studies applying MIF mutants with the CXXC cysteines exchanged indicated that the CXXC motif or the presence of the cysteines was at least partially necessary for the GCOR activity of MIF. In those studies, no direct proof for an involvement of a redox process was provided, thus leaving it open whether the reduced activity of these mutants was caused by a lack of redox activity or by subtle conformational alterations of the MIF molecule that abolished receptor binding or other MIF-target protein interactions. The observation that the cysteine mutants retained a significant GCOR rest activity of over 30%, also indicated already that additional other stimulatory pathways for MIF-mediated immune cell activation would exist. Together, the previous GCOR studies applying the cysteine and C-terminal mutants of MIF had initially prompted us to hypothesize that CXXC-spanning MIF-derived fragments might retain some GCOR activity and possibly other immunological and cellular activities of MIF. Our current study confirms this assumption and demonstrates that the CXXC-spanning MIF-derived peptide MIF-(50–65), which consists of only 16 residues, can in fact mimic certain MIF activities.

MIF-(50–65) encompasses residues 50–65 of the MIF sequence. According to the x-ray crystallographic data of human MIF (17), the sequence stretch 50–65 comprises a random coil element followed by a loop, which connects the random coil region with the β4-strand of MIF. In full-length MIF, the random coil region is preceded by the β2- and β3-strands. Our far-UV studies demonstrated that MIF-(50–65) had substantial secondary structure. Reduced MIF-(50–65) was found to be mainly in a random coil conformation. A minimum at 225–227 nm was also present indicating that the reduced form of MIF-(50–65) had a β-turn-forming propensity. Upon oxidation, the portion of β-turn conformation strongly increased as indicated by the absolute increase of the minimum at 225–227 nm. At the same time, the fraction of random coil was markedly diminished. This suggested that disulfide bridge formation induced a β-turn stabilization that was associated with a conformational ordering of the molecule (55). Of note, the side chain-to-side chain lactam-bridged analog of MIF-(50–65) was found to have a strong β-turn conformation. As this analog was designed to form covalent cyclization at the same residues and with the same ring size as for the disulfide-cyclized peptide, it is clear that disulfide-mediated oxidation of the peptide is associated with β-turn stabilization. Conformational ordering might include states consisting of β-strand-β-turn-β-strand or of β-strand-β-turn-α-helix. The latter one is a characteristic motif for the active site of various TPOs. No data on the conformation of the oxidized state of MIF have been available until now. According to the former model, oxidized MIF-(50–65) would contain an antiparallel β-sheet, with the connecting turn conformation and entire structure stabilized by the disulfide bond between Cys57 and Cys80. Oxidation-based disulfide formation

middle right panels). C57S/C60S-MIF-(50–65) (Ser-MIF-(50–65)) was almost inactive. Side chain-to-side chain-cyclized MIF-(50–65) (cyclo-MIF-(50–65)) was analyzed in comparison to a linear control peptide to probe for the importance of the β-turn conformation and showed comparable activity as wild-type MIF-(50–65) (lower panel). B, pull-down analysis demonstrated that MIF-(50–65) directly bound to JAB1. Complexes of biotinylated MIF-(50–65) and in vitro translated radiolabeled JAB1 were precipitated with streptavidin-conjugated magnetic beads and bound peptides/proteins analyzed by 13.5% SDS-PAGE and autoradiography as described under “Materials and Methods.” An autoradiograph showing radiolabeled JAB1 bands is shown. The biotinylated bis-serine variant of MIF-(50–65) complexed JAB1 to an almost equal degree as MIF-(50–65). Control incubations to account for nonspecific binding were performed without the biotinylated peptides (radiolabeled JAB1 + beads alone = control). For control purposes, Western blots of the pull-downs were performed and the biotinylated peptides stained with peroxidase-conjugated streptavidin (pull-down/blotting control; not shown). The autoradiograph shown is representative of 3 independent gels. C, both MIF-(50–65) and C57S/C60S-MIF-(50–65) induce p27 extraction. Control incubations encompassed incubation of cells alone and treatment of cells with the volume of phosphate buffer (NaP) used in the incubations with the highest peptide concentration. Gel loading was standardized for equal protein concentrations by Bradford analysis. The blots shown are representative of 3–4 independent experiments.
and structural stabilization of MIF-(50–65) was confirmed by the near-UV data, which showed the formation of a maximum between 270 and 285 nm, typical of disulfide bonds. Oxidation-mediated intramolecular disulfide formation in MIF-(50–65) was also verified by mass spectrometric analysis, which showed that the oxidized peptide had a mass that was almost precisely 2 mass units lower than that of the reduced peptide, whereas the obtained fraction of MIF-(50–65) dimers with intermolecular disulfides was negligible. Stabilization of β-turn formation in oxidized MIF-(50–65) was further confirmed by the CD data obtained for the bis-serine variant of MIF-(50–65). Although NMR studies will ultimately be necessary to obtain detailed information about the conformation stabilized in MIF-(50–65), our CD data obtained for reduced and oxidized MIF-(50–65), the bis-serine mutant, and the side chain-to-side chain lactam-bridged analog together indicate that MIF-(50–65) contains a β-turn-stabilized conformation, likely a β-strand-β-turn-β-strand conformation. Of note, significant β-turn stabilization and conformational ordering via disulfide bridge formation and synthetic constraints has been described previously also for hexa- and octa-peptides containing the CXXC active sites of the TPORs PDI, Grx, Trx, and Trr (35–37).

We found that MIF-(50–65) had a strong reducing potential with an $E' \theta$ value of approximately $-0.258$ V. Although the $E' \theta$ value of full-length MIF is unknown, such an electronegative $E' \theta$ value was surprising as it is among the most negative values found for TPORs. For example, the $E' \theta$ value of Trx is $-0.270$ V and that of Trr is around $-0.26$ V. It is therefore unlikely that the $E' \theta$ value of MIF is significantly more electronegative than that of the MIF peptide. In fact, we propose that the value of MIF-(50–65) may be taken to get a first approximation of an $E' \theta$ value of MIF. Given that the $E' \theta$ value of MIF might be in the range of approximately $-0.25$ V, the value for MIF-(50–65) would have been predicted to be more oxidizing, as Moroder and colleagues (35, 36) have shown for most TPORs, that the derived peptide fragments had much more oxidizing redox potentials. For example, linear octa-peptides covering the CXXC region of Trx and Trr were 60–80 mV more oxidizing than the full-length proteins. This effect was even more pronounced when TPOR-derived hexapeptides were cyclized by covalent N → C bonds. Under such enhanced turn-stabilizing conditions, only the PD-derived peptide had an $E' \theta$ value that was within the range of that of full-length PDI, whereas the values of the bis-Cys-cyclo peptides derived from Grx, Trx, and Trr were drastically more oxidizing than the full-length proteins (35, 36).

The strong reducing potential observed for MIF-(50–65) implies that the MIF peptide has a strong tendency to become oxidized. The $E' \theta$ potential of MIF-(50–65) as compared with the more oxidizing potentials of other TPOR-derived CXXC-containing peptides could be due to the greater length of the MIF-derived peptide. MIF-(50–65) contains 16 residues as compared with the reported 6–8 residues of the peptides of the TPORs. In fact, the CD data indicated a significant turn and, as mentioned, possibly an intramolecular β-sheet-forming propensity, an observation that was in line with the secondary structure prediction according to Garnier (54). Although probably (co-)induced by disulfide formation, the sheet-forming propensity of the peptide could be further assisted by long range interactions in MIF-(50–65), i.e. the ends of either β-strand, and could itself in turn facilitate disulfide formation between Cys residues 57 and 60.

In addition, the His residue at position 63 and the neighboring serine could assist disulfide formation by abstracting a proton from Cys$^{72}$ or Cys$^{49}$ or by stabilizing thiolate anion, respectively, with the resulting thiolate anion being able to exert a nucleophilic attack onto the other Cys thiol group. Neighboring His residues have been implicated in influencing the disulfide-forming capacities of CXXC cysteines of TPORs (56). On the other hand, neighboring His side chains could favor the formation of mixed disulfides. Whereas MIF-(50–65) forms mixed disulfides with both glutathione and cysteine, the equilibrium fractions of the mixed disulfides are much smaller than for the reported Trx and PDI peptides (35). Among several mechanistic possibilities, this could mean that the His side chain could be involved in both the formation and cleavage of the mixed disulfides.

Only the mixed disulfides of MIF-(50–65) with glutathione occurred in significant amounts at equilibrium. In contrast, the mixed disulfides with cysteine were short living and transient. Compared with peptide fragments of the other TPORs (35), the fraction of mixed disulfides formed by MIF-(50–65) was small. This finding is consistent with the measured negative $E' \theta$ value of MIF-(50–65).

S-Thiolation of MIF-(50–65) could be indicative of a potential S-thiolation of full-length MIF in the cell, which in turn may have functional relevance in cellular redox regulation. Interestingly, glycosylation-inhibiting factor (GIF), a mediator homologous to MIF, has been shown to be cysteinylated at Cys$^{60}$ and cysteinylation of GIF has been suggested to represent a functional post-translational modification of GIF (63). Trx was recently demonstrated to become glutathionylated under conditions of oxidative stress. Glutathionylation was detected to occur at Cys$^{72}$ of Trx but not at the CXXC cysteine residues (64). Given this latter observation made for Trx, it is therefore unclear whether glutathionylation of MIF-(50–65), which occurred at Cys$^{57}$, Cys$^{60}$, or both CXXC Cys residues, could be an indication for glutathionylation of MIF in the cell. Nevertheless, the data obtained in our study in conjunction with the reports on the cysteinylation and glutathionylation of GIF and Trx, respectively, warrant further studies addressing the issue of a potential S-thiolation of cellular MIF. MIF-mediated class II antigen processing (21) could also involve S-thiolation of MIF as a transition state.

The strongly negative redox potential of MIF-(50–65) suggests that the redox activity could be critical for the biological activities observed for the peptide. Although this is possible and, given our knowledge on the connection between the redox activity of full-length MIF and its immunological effects (19, 22, 23), even likely, the current study gives no final proof for this notion, because the applied bis-serine variant was not found to be inactive in all biological assays performed. For example, C57S/C60S-MIF-(50–65) bound to JAB1 similar to MIF-(50–65) and exhibited p27-stabilizing properties comparable with the wild-type peptide. Moreover, the observation that cysteine-less cyclo-MIF was able to activate ERK phosphorylation indicated that for at least some MIF target proteins, disulfide formation could rather have conformational functions (see proposed molecular model below).

The native-like biological activity of MIF-(50–65) was demonstrated using a number of relevant biological assays. Glucocorticoid overriding has been shown to be a unique property of MIF and represents an important mechanistic principle through which MIF can exert its specific pro-inflammatory spectrum of activities (3). The observation made in this study that the MIF peptide exhibited GCOR activity is therefore probably the most important finding to indicate that MIF-(50–65) can exert native-like MIF activities. Enhancement by MIF of cell proliferation has also been widely demonstrated, and this activity of MIF has been suggested to be associated with a role of MIF as a tumor growth promoter (5). We applied a cell proliferation assay based on the use of primary cells. MIF-(50–
In contrast, a tenth of this concentration (330 nM) resulted in growth-stimulatory activity was obtained with bFGF- and VEGF-stimulated MVECs. Because primary cells were used for this assay and because MIF has been implicated in endothelial cell regulation and atherogenesis (15), this result further demonstrated the relevance of the activity profile found for the peptide.

MAPK activation by MIF and modulation of JAB1 pathways have been among the very few molecular machineries identified for MIF signaling to date. We used assays addressing either activity branch in our study. Quite surprisingly, MIF-(50–65) not only led to phosphorylation of ERK1/2 but also was found to stimulate this activity within a few minutes. This indicated that the peptide could have acted as an agonist for the putative MIF receptor.

It has been demonstrated that MIF can be taken up by target cells through an apparently receptor-independent endocytosis pathway. As mentioned, the fast mode of action on ERK1/2 activation which MIF-(50–65) shares with MIF argues, however, that the peptide directly acts on a cell surface protein to trigger a typical, fast signaling event. By contrast, peptide activity on p27 stabilization could occur via a different, independent pathway, as these experiments were performed for several hours. Endocytosis studies showed that both MIF-(50–65) and the bis-serine variant are taken up by typical MIF target cells and that uptake was less efficient than that for full-length MIF. Significant and comparable endocytosis could be measured for both peptides and occurred within 60 min, suggesting that the different structure activity profiles of MIF-(50–65) and its serine variant are not due to different endocytosis rates. It is currently unclear how endocytosis of the peptides and signaling are connected. Also it is not yet clear why uptake of MIF is more efficient. As the difference in the fluorescence labeling ratio can probably not fully account for the different uptake rates between MIF and the peptides, we conclude that mechanistic differences may exist. The concentration and kinetic profiles of the MIF and peptide activities in the various assays applied do not yet allow for a unifying mechanistic picture in discerning receptor-mediated versus intracellular signaling-mediated and CXCXC-based versus sequence-based effects. Detailed kinetic studies on MIF and the peptides in comparison to cyclo-MIF-(50–65) and other analogs will have to be performed in the future to further clarify this point.

The most active concentrations of MIF-(50–65) were different over the assays performed. Optimal GCOR and MVEC growth-stimulatory activity was obtained with ~3 μM peptide. In contrast, a tenth of this concentration (330 nM) resulted in maximal induction of ERK1/2 phosphorylation. The peptide concentrations necessary for the biological effect to be observed were lower than those of full-length MIF throughout, but although the potency of the peptide was ~1000-fold lower in the GCOR assay, the activities of MIF and the peptide were within the same range in the MAPK activation and the p27 stabilization assays (~4–40- and 3-fold difference, respectively). The observed differences between the assays could well represent the different molecular pathways addressed, as also the optimal concentrations of MIF varied. For example, micromolar concentrations of MIF have been suggested previously (5, 6) to be associated with the enzymatic effects of MIF. Also, different endocytosis efficiencies could underlie these differences, as rMIF was observed to be endocytosed at a much stronger rate.

Given its in vitro catalytic activity and its MIF-like immunological and cellular activities, as demonstrated in this study, MIF-(50–65) represents a dramatically minimized MIF analog of only 16 amino acids compared with the 114 residues of full-length MIF. Although this minimization is dramatic and surprising, protein minimization efforts have recently led to the identification of a number of small peptides, domains, and motifs that are able to cover a significant spectrum of the activities of the parent proteins. As discussed above, a number of TPOR-derived peptides of a little as 6 residues have been described. However, so far these peptides have only been shown to share the catalytic oxoreductase and refolding activity with the full-length TPORs (35, 36). A 12-amino acid-long synthetic peptide, designed and selected as an Mdm2-binding protein, was shown to bind to the p53 binding pocket on the Mdm2 protein. The mini Mdm2-binding protein, which apparently effectively competes with the native protein, stabilized p53 and led to an activation of the p53 response (65).

Although some of the biological activities measured for MIF-(50–65) were obtained by peptide concentrations that were within the range of those used for rMIF, i.e. 3 μM of peptide had about the same MVEC-stimulatory effect as 400 nM rMIF, overall the peptide seemed to have a lower potency than full-length MIF in most assays performed in this study. However, this was not surprising as small peptide sequences, due to their lack of three-dimensional structure, often show reduced affinities and activities compared with their parent molecules. For example, a minimized transforming growth factor-α peptide, truncated by 8 out of 50 residues, had a 10-fold lower affinity for the receptor than the native molecule (66). A mini-proinsulin molecule consisting of one chain shows native foldability but only had a 1500-fold lower receptor binding affinity and had no biological activity (67).

There are at least two examples in the literature demonstrating striking protein minimization with the resulting minimized molecules exhibiting potent native-like activities, although their sequence/structure is not found in the sequence of the native molecules. A 14-residue peptide was selected by phage display as a potent agonist of erythropoietin that could stimulate erythropoiesis. The signaling pathways activated by this erythropoietin agonistic peptide appeared to be identical to those induced by the native hormone (68). Arkil et al. (69) recently identified a non-peptidyl small molecule, which bound to the cytokine interleukin-2 through mimicking the protein-protein interface that the interleukin-2 receptor-a-subunit shares with interleukin-2. The small molecule was able to behave adaptively in binding to the predicted dynamic protein interface. This work as well as studies on the characterization of ligand-receptor interfaces such as those of the growth hormone-growth hormone receptor extracellular domain (70) have demonstrated that ligand receptor interfaces sufficient for receptor activation can be as small as to only cover a few amino acid residues. Thus, the 16-residue-comprising MIF peptide may well provide sufficient “conformational properties” to make up for a receptor-binding epitope to or that represent a docking site for a redox protein target of MIF or to represent the MIF-docking site for JAB1 binding (14). Discrete folding of mini-proteins or mini-domains has been demonstrated for a number of proteins (71), further confirming that MIF-(50–65) could be discretely folded to exert native-like activities on target proteins.

Nevertheless, the current study leaves it open through which molecular mechanism MIF-(50–65) exhibits its MIF-like biological activities. In fact, the 16-mer peptide could act via different pathways. It could bind to a cell surface receptor and modulate receptor activity through this binding event. In favor of such a mechanistic concept are structural analyses of MIF, indicating that the region around residue Cys65 is surface-exposed (17). On the other hand, our current study in conjunction with previous data (14) indicates that, in addition, the peptide could bind to JAB1, resulting in the modulation of JAB1-mediated MIF-regulatory pathways. Another intriguing
mechanistic possibility is suggested by a recent report (21) showing that MIF interacts with MHC II molecules and can act to process class-restricted peptides in a disulfide-mediated manner. Accordingly, MIF and thus the MIF-derived CXXC-spanning peptide could act through the MHC II machinery.

The observed conversion of reduced MIF-(50–65) from a mostly unordered molecule containing a predominant fraction of random coil structure into an oxidized species with marked β-turn structure, indicated that the MIF peptide could potentially dock onto target proteins such as insulin, JAB1, or the putative receptor. Such a binding event could initially involve a mainly unordered conformation, and upon binding or oxidation, the peptide could become ordered. As mentioned, such a mechanism has been proposed to be part of the redox mechanism of Trx (39), a TPOR enzyme that does not contain a helix-loop-strand Trx-fold. Our initial bioactivity data obtained using recombinant MIF-(50–65) exhibited MIF-like activities, mainly unordered conformation, and upon binding or oxidation, the peptide could become ordered. As mentioned, such a mechanism has been proposed to be part of the redox mechanism of Trx (39), a TPOR enzyme that does not contain a helix-loop-strand Trx-fold. Our initial bioactivity data obtained using recombinant MIF-(50–65) exhibited MIF-like activities.

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