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Structure activity studies of the cytokine macrophage migration inhibitory factor (MIF) reveal a critical role for its carboxy terminus

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Abstract Carboxy-truncated mutants of human MIF (MIF(1-104) and MIF(1-109)) were used in structure activity studies. CD spectroscopy revealed an overall structural similarity between the mutants and MIF. Denaturant-induced unfolding demonstrated that the C-terminus contributed significantly to the conformational stability of MIF. This appears to be due to the formation of two C-terminal β-strands. The mutants were enzymatically active, exhibiting half of the enzymatic redox activity of MIF. However, immunological analysis showed that deletion of both 5 and 10 C-terminal residues resulted in loss of the macrophage activating properties of MIF, providing functional evidence that the C-terminus is important for immunological activity and trimer formation. A more detailed study of the C-terminus may assist in identifying the molecular basis for the immunological and enzymatic activities of MIF.

Key words: Macrophage migration inhibitory factor; Cytokine; Protein structure; Mutagenesis; Structure activity study

1. Introduction

The classical T cell factor macrophage migration inhibitory factor (MIF) was re-defined recently as a pituitary hormone and macrophage-derived factor with pro-inflammatory properties [1–4]. The importance of MIF as T cell cytokine was confirmed [5]. MIF was found to be unique in counter-regulating the immuno-suppressive and anti-inflammatory effects of glucocorticoid hormones [6]. Of note, MIF was demonstrated to play a critical role in a number of pathologic conditions such as septic shock, physiological stress, or certain immune-mediated diseases [1,6–9]. Several lines of evidence indicate that MIF is one of few cytokines that may act by additional enzymatic functions [10–13]. However, the physiological enzymatic activity of MIF is not yet known.

The three-dimensional structure of MIF was elucidated recently by X-ray crystallography and NMR methods and MIF was found to be a trimer of identical subunits [12,14–17]. Each monomer contains two antiparallel α-helices that pack against a four-stranded β-sheet. In the trimer, the three β-sheets are arranged to form a barrel containing a central solvent-accessible channel (Fig. 1A). One structural feature that has not been characterized precisely is the C-terminal region of MIF. In particular, it is not clear if the C-terminus is composed of a β-strand configuration, or if it is structurally undefined. For example, no structure was found for the C-terminus of rat MIF [12], whereas for human MIF, none, one, or two short C-terminal β-strand elements have been proposed [14–17] (Fig. 1B). It has been suggested that the C-terminal residues of one monomer interact with residues located within a β-strand of an adjacent subunit to stabilize the trimer [14].

Although the elucidated structure of MIF is unique among cytokines and hormones, the barrel-like structural architecture of the MIF protein was found to be highly homologous to two bacterial tautomerases [12,18], indicating that trimer formation may be necessary for the suggested enzymatic activity of MIF. To resolve the issue of the C-terminal structure of MIF under solution conditions and because of the proposed importance of the carboxy terminus for MIF trimer stabilization and enzymatic activity, we generated carboxy-truncated mutants of human MIF and performed structure activity studies.

2. Materials and methods

2.1. Materials

Miscellaneous chemicals and enzymes were bought from Sigma-Aldrich Chemicals (Deisenhofen, Germany) and were of the highest grade commercially available. Dye terminator cycle DNA sequencing kits were from Perkin Elmer-Applied Biosystems Inc. (ABI) (Weiterstadt, Germany) and plasmids were from Novagen (Madison, WI, USA). All other molecular biology reagents including oligonucleotide primers were acquired from Gibco BRL (Eggenstein, Germany) or New England Biolabs GmbH (Heidelberg, Germany).

2.2. Site-directed mutagenesis and cloning of the carboxy-truncated mutants

Human MIF was amplified originally from Jurkat H33HJ-JA1 T cell DNA and cloned into the pET11b expression vector as described previously [4]. C-terminal deletion mutants of MIF were cloned from huMIF-pET11b by DNA amplification. Polymerase chain reactions (PCR) were performed in a Perkin Elmer 480 thermocycler (Perkin Elmer-ABI). PCR conditions were: 6 min at 94°C followed by 35 cycles of 70 s at 96°C, 45 s at 50°C, and 30 s at 72°C using 2 units of Vent polymerase (New England Biolabs). Primers were designed with NdeI (5′-GCT AGC GCA TAT GCC GAT GTT CAT CGT AAA CAC-3′) and BamHI (5′-CGG GAT CTT TAG TTC CAG CCC ACA TTG GCC G-3′ and 5′-CGG GAT CCT TAG GCC GCG TTC ATG TCG TAA TAG-3′ for MIF(1-109) and MIF(1-104), respectively) cloning sites and PCR products cloned into the NdeI and BamHI sites of pET11b. Mutant DNA sequences were confirmed by bidirectional sequencing of the clones using an ABI PRISM 377 DNA sequencer (Perkin Elmer-ABI).

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Abbreviations: MIF, macrophage migration inhibitory factor; wt, wild-type; huMIF, human MIF; CD, circular dichroism spectroscopy; HED, hydroxyethyldisulfide; GdnHCl, guanidinium hydrochloride

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2.3. Bacterial expression and purification of the mutant proteins

Expression and purification of the mutants was performed using a slightly modified protocol of that established for wild-type human MIF (wtMIF) [4]. Briefly, transformed *E. coli* BL21 (DE3) containing the mutant expression plasmids, were grown at 37°C until the optical density at 600 nm reached 0.6–0.8. Isopropyl 1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM and the incubation continued for an additional 3 h. Bacteria were harvested by centrifugation and the cell pellets frozen at −20°C until use. Bacterial pellets were resuspended in 3 ml of Tris-buffered saline (30 mM Tris-HCl, 100 mM NaCl, pH 7.2) and the cells disrupted in a French Press at 1240 psi. The bacterial extract then was centrifuged at 38 000×*g* for 30 min, the supernatant recovered, and sterile-filtered. Purification by anion exchange and C8-SepPak reverse-phase chromatography, and renaturation of acetonitrile-denatured protein was performed as described previously [4] except that a HQ column (Boehringer Mannheim, Mannheim, Germany) was used for the anion exchange chromatographic separation. Renatured mutants were kept at 4°C until use. One mutant, MIF(1-104), was found to aggregate more quickly than wtMIF, and thus was not used for longer than 2 weeks after renaturation.

2.4. Biochemical characterization of the mutants

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described [4] and was employed to verify expression of the mutant proteins. MIF(1-109) migrated at a size of approximately 12 kDa and MIF(1-104) was found to have a molecular weight of about 11.5 kDa (not shown). Size exclusion chromatography was performed on a Bio-Sil SEC 125-5 column (300×7.8 mm, Biorad, Munich, Germany) at a flow rate of 1 ml/min using 50 mM sodium phosphate buffer (pH 7.2) in the presence or absence of 7 M guanidinium hydrochloride (GdnHCl). Size markers were from Biorad and were chromatographed under the same conditions. As established previously for wtMIF [4,19], both mutants chromatographed at a molecular weight of 13 kDa in the presence of GdnHCl and at a size of approximately 25 kDa under denaturant-free elution conditions. Identity of the mutant proteins was verified further by matrix-assisted laser desorption ionization (MALDI) mass spectrometry using a Kratos Kompact MALDI III machine (Shimadzu, Duisburg, Germany). The experimentally determined masses for wtMIF (12347 Da), and the mutant proteins MIF(1-109) (11823 Da) and MIF(1-104) (11241 Da) were in good agreement with the theoretical, predicted MH^+ average mass values of 12346 Da, 11824 Da, and 11253 Da, respectively.

2.5. Conformational analysis and structural stability analysis

Circular dichroism spectropolarimetry (CD) spectra were recorded in a J700 spectropolarimeter (Jasco Inc., Tokyo, Japan) that was equipped with a thermostated cell holder. The spectra represent the average of three scans recorded at 25°C in the range between 195 and 250 nm and were collected at 0.5 nm intervals with a bandwidth of 1 nm and a time constant of 8.0 s. Cylindrical quartz cells with a path length of 10 mm were used in all experiments. Final protein concentrations were 1 μM in 20 mM sodium phosphate buffer (pH 7.2). CD spectra are presented as a plot of the mean molar ellipticity per residue ([Θ], deg cm^2 $dmol^{-1}$) versus the wavelength. Secondary structure analysis of the CD spectra was performed by the computer programs CONTIN [20,21], LINCOMB together with the data sets of Perczel et al. [22], Yang et al. [23], and Brahms and Brahms [24], and SELCON [25,26].

Guandinium hydrochloride (GdnHCl)-induced denaturation studies were performed by recording the mean molar ellipticity per residue as a function of wavelength (210–250 nm) and GdnHCl concentration as described previously [4,27]. Unfolding curves were expressed as the percentage of unfolded protein relative to native protein (i.e. the change in ellipticity at 222 nm) over the concentration of GdnHCl. GdnHCl at concentrations above 3 M did not interfere with the measurement at this wavelength. The free energy of unfolding was derived from this curve by using the equation:

$$\Delta G_d = -RT\ln[(y_n - y)/(y - y_d)],$$

where y is the ellipticity value at 222 nm and y_n and y_d are the ellipticities at 222 nm for the native and denatured states of the protein, respectively. The obtained values for ΔG_d were fitted to the equation:

$$\Delta G_d = \Delta G_d^{H_2O} - m[\text{GdnHCl}],$$

where $\Delta G_d^{H_2O}$ is the value of ΔG_d at zero denaturant concentration and m represents the slope of the curve that is a measure of the dependence of ΔG_d on the concentration of GdnHCl [28].

2.6. Biological assays

Enzymatic activity of the mutants was determined by the hydroxyethyldisulfide (HED) transhydrogenase assay following the method described by Holmgren [29] and that was established as an enzymatic assay for human MIF (to be published in detail elsewhere) [13]. Briefly, reduction of HED by reduced glutathione was measured in a MIF-catalyzed reaction. Oxidation of NADPH by oxidized glutathione was then measured in a coupled step and was recorded spectrophotometrically at a wavelength of 340 nm. Wild-type mouse and human MIF were determined to have a specific activity of 420 and 190 mU, respectively. Carboxy-truncated mutants were tested at the conditions established for wtMIF and their enzymatic activities compared to that of wild-type human MIF.

Since MIF was recently defined as a cytokine activating macrophages to kill *Leishmania major* parasites (Juettner et al., manuscript in preparation) the leishmanicidal activity of the MIF mutants was tested [30]. Briefly, thioglycolate-elicited murine peritoneal exudate cells were washed twice and resuspended in C-RPMI culture medium 1640 (Biochrom, Berlin, Germany, supplemented with 2 mM L-glutamine, 10 mM HEPES buffer, 7.5% $NaHCO_3$, 0.05 mM 2-ME, 100 μg/ml penicillin, 160 μg/ml gentamycin and 10% selected FCS with a total LPS content < 600 pg/ml), seeded into Labtek tissue culture chambers (2×10^5/chamber; Flow Laboratories, Meckenheim, Germany) and were allowed to adhere for 3–4 h (at 37°C, 5% CO_2, 95% air humidity); thereafter, non-adherent cells were removed by three extensive washings with culture medium. Prior to infection, macrophage cultures were incubated for 4 h either in culture medium alone or in culture medium with 1 μg/ml of MIF or the MIF mutants. Addition of 20 ng/ml interferon-γ served as an internal positive control. The macrophages were then infected with *L. major* promastigotes (parasite:cell ratio=10:1) for 4 h in the respective medium. Thereafter, non-phagocytosed parasites were washed off, and the cultures were further incubated in the respective medium for 72–96 h. Intracellular live amastigotes were then assessed after staining with a solution of ethidium bromide (50 μg/ml) and acridine orange (5 μg/ml) by fluorescence microscopy at 450–490 nm. The percentage of infected macrophages (infection rate) and the number of parasites/100 infected macrophages (mean ± S.E.M. of 6 observations on three cultures in each experiment) were determined. Differences between treated vs. control cultures were tested for statistical significance by Student's *t*-test for unpaired samples (two-tailed).

3. Results

Two carboxy-truncated mutants of huMIF with 5 and 10 residues deleted, respectively, were constructed by site-directed mutagenesis and expressed in *Escherichia coli*. The mutants were designed to have the putative C-terminal β-strand(s) β_7 MIF(1-109) or $\beta_6+\beta_7$ (MIF(1-104)) deleted (Fig. 1B and C). Mutants were purified to homogeneity essentially by the method used for wtMIF [4]. Far-UV CD, which is an excellent method for analyzing the conformation of proteins in solution [31], was employed and confirmed the overall structural similarity between the mutants and wtMIF (Fig. 2A). Detailed spectral analysis showed that both wtMIF and the mutants had a broad negative ellipticity between 205 and 225 nm and a positive ellipticity at 197 nm. Although the spectra were almost identical, a small shift towards shorter wavelengths was seen in the spectra of both mutants, and was found to be consistent with an increase in random coil conformation. Next, quantification of the secondary structure composition of the mutants in comparison with wtMIF was performed by employing a variety of computational deconvolution methods (Table 1). This analysis confirmed

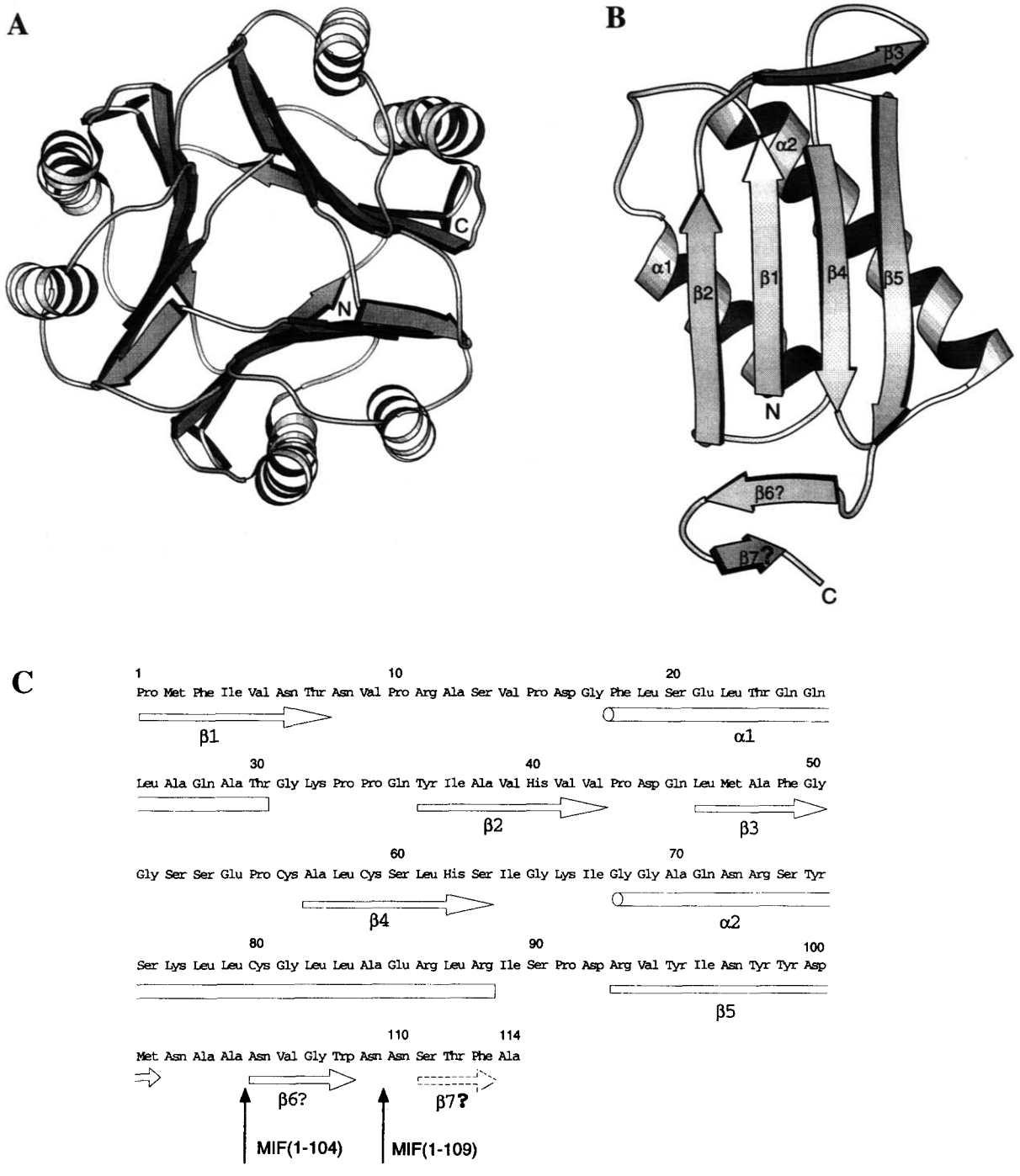

Fig. 1. Structure of human MIF. A: Architecture of the MIF trimer. The view is along the molecular three-fold axis. B: Ribbon structure of the MIF monomer [34]. C: Primary sequence and secondary structure of the MIF monomer. Open arrows indicate β-strands and cylinders represent α-helices. The sites of the deletions are indicated by closed arrows. The putative presence of the C-terminal β-strands 6 and 7 is expressed by question marks. β_7 is depicted by a dotted arrow.

that the solution conformation of both mutants was very similar to that of wtMIF.

An increase in random coil structure was predicted by most of the deconvolution methods. According to methods 1–3 and 5 this increase was caused by deletion of residues 110–114, whereas no further increase was seen upon deletion of residues 105–109.

The alterations in random coil structure were paralleled by a decrease in β-sheet conformation, when the mutants were compared to wtMIF. Moreover, most deconvolution methods (algorithms 1, 3, and 5) predicted that deletion of residues 109–114 is sufficient for the observed reduction in β-sheet conformation, whereas no further decrease was observed when MIF was truncated at position 104. Of note, deconvolution by convex constrained analysis using the data set by Perczel et al. [22], which is an excellent method to estimate antiparallel and parallel β-sheet components [31], indicated that residues 109–114 were in an antiparallel β-sheet conformation. Analysis of the β-turn component by algorithms 2, 3, and 5 but not algorithm 4 predicted a decrease in β-turn

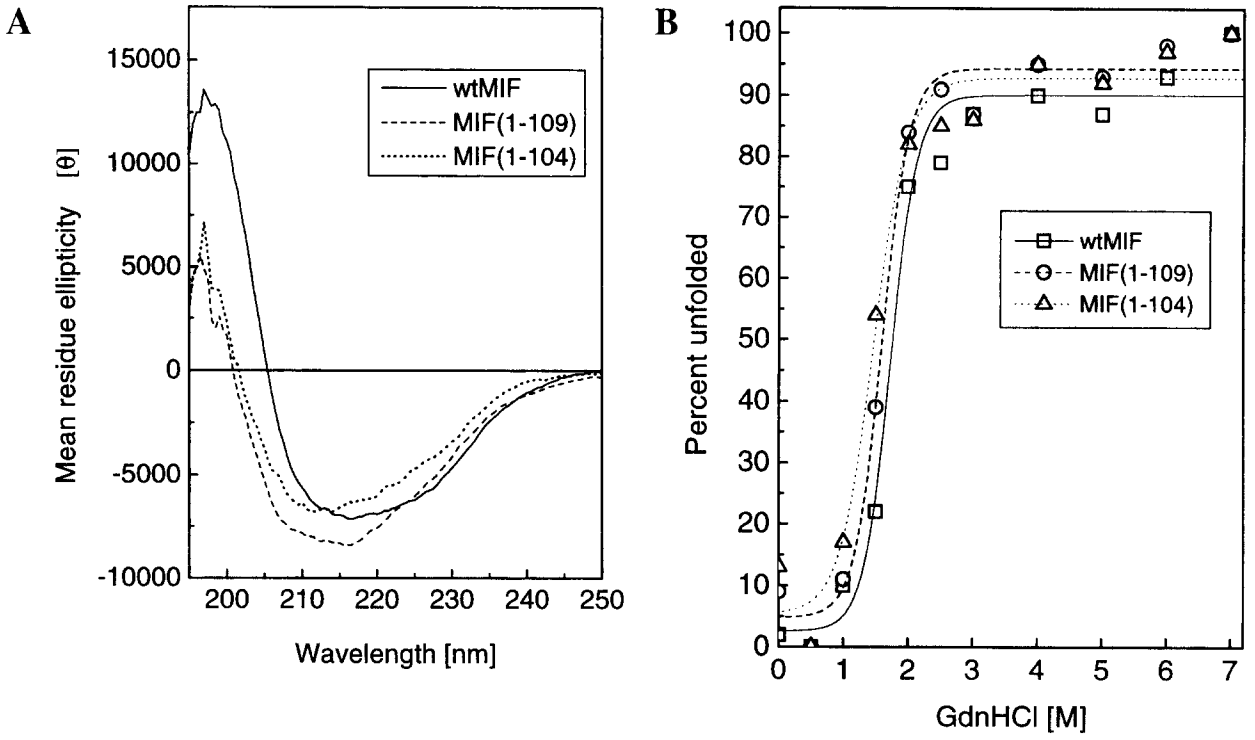

Fig. 2. Far-UV CD spectroscopy of wtMIF and the mutants. A: CD spectra of wtMIF, MIF(1-109), and MIF(1-104). CD spectra were recorded at a concentration of 1 μM in 20 mM phosphate buffer, pH 7.2. B: GdnHCl-induced unfolding of wtMIF and MIF(1-109) and MIF(1-104). Unfolding curves are expressed as the percentage of unfolded protein relative to native protein (i.e. the change in ellipticity at 222 nm) over the concentration of GdnHCl. CD spectra and unfolding experiments were performed as described in Section 2.

structure in the larger mutant, with no further decrease seen upon deletion of residues 104–109. Analysis of the α-helical portions by the various methods did not provide conclusive data. Overall, no significant alterations in α-helical content were observed between wtMIF and the mutants.

To further investigate the effect of the carboxy terminus of

Table 1
Quantitative analysis of the secondary structure contents of the mutants in comparison to wild-type MIF[a]

Method	Secondary structure content (%)						
	α-helix	β-sheet	Parallel β-sheet	Antiparallel β-sheet	Parallel β-sheet/β-turn	β-turn	Unordered
1							
wtMIF	13	NA	NA	45	22	NA	21
MIF(1-109)	7	NA	NA	27	27	NA	36
MIF(1-104)	11	NA	NA	26	30	NA	34
2							
wtMIF	25	30	NA	NA	NA	33	12
MIF(1-109)	19	28	NA	NA	NA	23	30
MIF(1-104)	22	26	NA	NA	NA	24	28
3							
wtMIF	11	50	NA	NA	NA	15	24
MIF(1-109)	18	34	NA	NA	NA	11	38
MIF(1-104)	10	43	NA	NA	NA	9	38
4							
wtMIF	24	NA	10	20	NA	22	25
MIF(1-109)	27	NA	6	20	NA	28	24
MIF(1-104)	30	NA	6	16	NA	26	22
5							
wtMIF	21	34	NA	NA	NA	21	24
MIF(1-109)	25	26	NA	NA	NA	12	38
MIF(1-104)	28	28	NA	NA	NA	14	31

[a]CD spectra were recorded as described in Section 2. Quantitative analysis of the spectra was performed by various deconvolution methods as described previously (see Section 2, [4]).
1, 2, 3: Calculation by the LINCOMB program based on the methods of Perczel et al. [22], Yang et al. [23], and Brahms and Brahms [24], respectively.
4, 5: Analysis by the CONTIN [20,21] and SELCON [25,26] computer programs.
NA: not applicable.

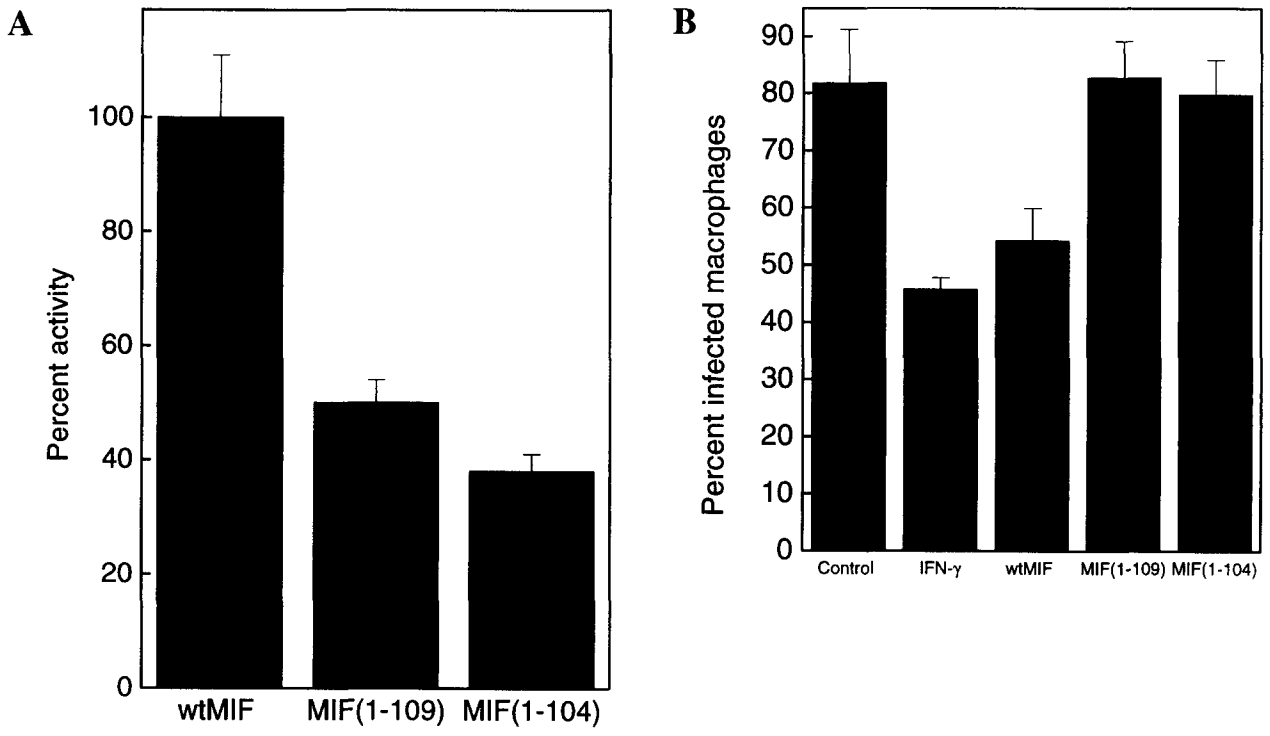

Fig. 3. Biological activity of the mutants in comparison with wtMIF. A: Enzymatic redox assay. The HED transhydrogenase activity of the mutants is expressed as percent activity of wtMIF. Data represent the mean ± S.D. of triplicate experiments. B: Macrophage activating activity of the mutants in comparison with wtMIF as determined in a macrophage-dependent *Leishmania*-killing assay. Activation of macrophages is expressed as percent infected macrophages. Control cells were not pretreated with cytokines. Addition of 20 ng/ml interferon-γ (IFN-γ) served as a positive control. Data represent the mean ± S.E.M. of triplicate experiments. Biological assays were performed as described in the experimental section.

MIF on trimer stabilization and conformational stability of the protein, the mutants were studied by CD together with GdnHCl-induced unfolding, and the results compared to the effect observed for wtMIF (Fig. 2B). Unfolding was found to be cooperative for both wtMIF and the mutants, with the midpoints of denaturation estimated to be at a concentration of 1.8 M GdnHCl for wtMIF, and at 1.6 M and 1.5 M for MIF(1-109) and MIF(1-104), respectively. The values for the free energy of unfolding in the absence of denaturant ($\Delta G_d^{H_2O}$) were determined by linear extrapolation of ΔG_d in the transition region [28]. The $\Delta G_d^{H_2O}$ value for MIF(1-109) was similar to that measured for wtMIF (12.51 kJ mol^{-1} versus 11.9 kJ mol^{-1}), whereas deletion of 10 C-terminal residues appeared to markedly reduce protein stability at zero denaturant concentration ($\Delta G_d^{H_2O}$ = 8.55 kJ mol^{-1} for MIF(1-104)). Both, the values for the midpoint of denaturation and $\Delta G_d^{H_2O}$ were found to be in excellent agreement with previously determined values for MIF [4].

To examine the effect of carboxy-terminal truncation on the functional properties of MIF, the mutant proteins were subjected to an enzymatic assay and an immunological assay that had been shown previously to be a good measure of the catalytic and macrophage activating activities of MIF, respectively ([13], Juettner et al., manuscript in preparation). MIF(1-109) showed a 50% reduction in enzymatic redox activity when compared to wtMIF. MIF(1-104) also was found to retain significant activity (40%) in this test, indicating that the C-terminus may not be involved directly in the observed catalytic activity (Fig. 3A). Finally, the immuno-functional importance of the carboxy terminus was studied in a macrophage test of *Leishmania*-killing. When compared to wtMIF and an internal positive control, both the larger mutant and the mutant that was truncated at position 104 did not show any remaining enhancement of *Leishmania*-killing by macrophages (Fig. 3B). This indicated that residues 104–114 may be critical for the immunological effects of MIF.

4. Discussion

Important progress has been made recently in understanding the biologic role of MIF [1,2,5–9]. These studies showed that MIF is an important cytokine that is critically involved in the regulation of the host immune and stress responses. With respect to the molecular properties, the recent elucidation of the three-dimensional structure of MIF [12,14–17] has added further to our understanding of the role of MIF. However, because a receptor for MIF has not yet been identified and because the identity of a catalytic center that is responsible for the enzymatic activity of MIF is unclear, the molecular mechanism of MIF action continues to be unknown.

Important insight into the molecular basis of MIF function can be expected from structure activity studies of the C-terminus of MIF. This region has been proposed to stabilize the trimeric structure of the molecule by the formation of hydrogen bonds between subunits [14]. Furthermore, a functional role for the C-terminus was suggested when a striking structural similarity between MIF and the bacterial enzyme 5-carboxymethyl-2-hydroxymuconate isomerase was discovered [12]. However, the precise structure of the carboxy-terminal region has not yet been resolved, with the various X-ray struc-

tures at variance and differences observed between the X-ray crystallographic and NMR data.

We employed carboxy-truncated mutants of MIF to resolve the solution structure of its C-terminus and to study the role of this region for the conformational stability and biological activity of MIF. Mutants were designed such that in one mutant, MIF(1-109), the putative β_7-strand was removed, and that in the other mutant, MIF(1-104), both the putative β_6- and β_7-strands were deleted. Hydrogen bonding-mediated stabilization of the trimer had been predicted to occur by the interaction of the β_6-strand of one subunit with the β-sheet of an adjacent monomer, leading to the appearance of a six-stranded β-sheet per monomer [14]. In another X-ray crystallographic report [16], it has been suggested that inter-subunit interactions in this region are preceded by the formation of an intramolecular antiparallel β-sheet involving the β_6- and β_7-strands of one monomer.

Overall, our analysis of the solution structure of the mutants by CD followed by estimation of the secondary structure contents showed that the mutants exhibit a decreased content in β-sheet structure. This was found to be paralleled mainly by a concomitant increase in random coil structure, indicating that the C-terminal residues contribute towards tertiary structure stabilization of MIF. This is in accordance with the crystallographic analysis of human MIF. Together with the X-ray data [16], our results as obtained by algorithms 1, 3, and 5 suggest that residues 111–113 form a β-strand in solution. Furthermore, deconvolution with these methods indicated that while deletion of residues 110–114 led to a loss in β-sheet structure, no further reduction in β-sheet content occurred, when the residues composing the β_6-strand, i.e. 105–108, were also deleted. Analysis of the CD spectra by algorithms 2 and 4 did not confirm this finding. However, methods 1 and 3 have been demonstrated to better predict the β-sheet portions in β-sheet-rich proteins [31], and the overall significance of the changes in structural contents appears to be higher for algorithms 1, 3, and 5. Only two of the methods allow for the differentiation between antiparallel and parallel β-sheet portions. Of these, the method by Perczel et al. (algorithm 1), which is especially suited for the analysis of β-sheet contents, was found to predict a marked decrease of antiparallel β-sheet structure upon deletion of residues 109–114, but did not reveal any further decrease, when MIF was truncated after residue 104. Although not confirmed by method 4, our data argue for the existence of an intramolecular antiparallel β-sheet within the C-terminal region of MIF. In conclusion, the results confirm the existence of the putative β_7-strand, and together with the evidence collected by Sugimoto et al. [16], would suggest that, in solution, this β-sheet is formed by the C-terminal β-strands 6 and 7, i.e. residues 105–108 and 111–113, respectively.

To determine the stabilizing effect of this structure for the trimer, we performed GdnHCl-induced unfolding experiments of the mutants. Significant differences in the conformational stabilities between the mutants and wtMIF were observed. Moreover, the mutants also were found to differ among each other. The midpoint of unfolding, which is a good measure of the structural stability of proteins, was within the same range for wtMIF and the mutants, and was found to be smaller than 2 M GdnHCl with a gradual decrease of stability seen, when the C-terminal region was truncated. As expected from previous studies on the stability of wtMIF [4], $\Delta G_d^{H_2O}$ was found to be small, i.e. considerably smaller than for most globular proteins, for both wtMIF and the mutants. The value for MIF(1-109) was similar to that of wtMIF (12.51 kJ mol−1 versus 11.90 kJ mol^{-1}), whereas additional deletion of residues 105–109 in MIF(1-104) led to a marked drop in $\Delta G_d^{H_2O}$ (8.55 kJ mol^{-1}). Together, analysis of the conformational stability of the mutants indicated that: (a) deletion of the entire C-terminus of MIF did not alter the overall stability of the protein and (b) deletion of both C-terminal β-strands led to a small but significant decrease in protein stability. The measured conformational stabilities do not readily correlate with the proposed deletion of β-strands. In this regard, one would have expected a more pronounced reduction in stability for mutant MIF(1-109), as this would lead to elimination of the C-terminal β-sheet. However, deletion of these residues may also lead to other molecular interactions that might act to over-compensate the assumed β-strand-related loss in stability. Also, it has been suggested that the trimer might not be the functional unit of MIF under physiological solution conditions ([19,32], Mischke et al., manuscript in preparation). Thus, it might be speculated that yet other conformations exist that have not been detected by X-ray crystallography or NMR, which are methods that both rely on the use of highly concentrated, non-physiological, protein solutions. We are currently pursuing an experimental approach to clarify this point and to further define the contribution of the C-terminus towards trimerization by covalent cross-linking and electrophoretic analysis of wtMIF and the C-terminal mutants.

Both mutants were found to exhibit significant enzymatic activity, indicating that C-terminal residues did not participate directly in the enzymatic redox activity of MIF. Rather, our data suggest that the observed reduction in enzymatic activity may be caused indirectly by the decreased conformational stability of the mutants. Because the precise molecular mechanism of the enzymatic redox and tautomerase activities of MIF is not known yet, no immediate conclusions can be drawn from our studies as to the effect of carboxy-truncation on tautomerase activity.

A macrophage activating activity is well established for MIF [2,4,6,33]. In this study, we employed a macrophage-based *Leishmania*-killing assay. In this test, MIF can function to enhance macrophage killing of *Leishmania* parasites in a concentration-dependent manner (Juettner et al., manuscript in preparation). The assay was employed in this study to assess the immunological or cytokine-like activity of the MIF mutants. Of note, neither MIF(1-109) nor MIF(1-104) exhibited any remaining macrophage activating properties. Thus, while no direct dependence of the enzymatic activity of MIF on the C-terminus was found, it appears that the C-terminal region is critical for MIF-mediated activation of macrophages. Because it is likely that these immunological activities of MIF occur by a receptor-mediated mechanism, our data argue that the C-terminus is involved in receptor binding.

In conclusion, our results suggest that the C-terminus is an important target for further structure activity studies of MIF. More detailed analyses by site-directed mutagenesis will be necessary to identify the exact residue or residues that are important in this region. Moreover, analysis of the C-terminus may assist in identifying the mechanism that may be the molecular basis for the immunological and enzymatic activities of

MIF. In addition, our data indicate that C-terminal deletion mutants of MIF could be a valuable tool in the design of minimized MIF molecules for potential therapeutic applications.

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