Specific reduction of insulin disulfides by macrophage migration inhibitory factor (MIF) with glutathione and dihydrolipoamide: potential role in cellular redox processes

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Received 8 May 1998

Abstract The molecular mechanism of action of MIF, a cytokine that plays a critical role in the host immune and inflammatory response, has not yet been identified. We recently demonstrated that MIF is an enzyme that exhibits oxidoreductase activity by a cysteine thiol-mediated mechanism. Here we further investigated this function by examining the reduction of insulin disulfides by wild-type human MIF (wtMIF) using various substrates, namely glutathione (GSH), dihydrolipoamide, L-cysteine, β-mercaptoethanol and dithiothreitol. The activity of wtMIF was compared to that of the relevant cysteine mutants of MIF and to two carboxy-truncated mutants. Only GSH and dihydrolipoamide were found to serve as reductants, whereas the other substrates were not utilized by MIF. Reduction of insulin disulfides by MIF was closely dependent on the presence of the Cys^{57}-Ala-Leu-Cys^{60} (CALC) motif-forming cysteines C57 and C60, whereas C81 was not involved (activities: 51 ± 13%, 14 ± 5%, and 70 ± 12% of wtMIF, respectively, and 20 ± 3% for the double mutant C57S/C60S). Confirming the notion that the activity of MIF was dependent on the CALC motif in the central region of the MIF sequence, the C-terminal deletion mutants MIF(1–105) and MIF(1–110) were found to be fully active. The favored use of GSH and dihydrolipoamide indicated that MIF may be involved in the regulation of cellular redox processes and was supported further by the finding that MIF expression by the cell lines COS-1 and RAW 264.7 was significantly induced upon treatment with the oxidant hydrogen peroxide.

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Key words: Macrophage migration inhibitory factor; Insulin disulfide reduction; Redox process

1. Introduction

Our knowledge about the classical T cell cytokine macrophage migration inhibitory factor (MIF) [1,2] was vastly improved by the recent findings that MIF is a pituitary hormone and macrophage-derived cytokine playing a critical role during the host inflammatory and stress response [3–6]. In this function, MIF was demonstrated to be a pro-inflammatory mediator of several inflammatory diseases including Gram-negative [3] and Gram-positive septic shock [7], and certain immune-mediated and autoimmune diseases [8–10]. Of note, MIF is the only cytokine to be induced by glucocorticoids and then serves to function as an endogenous counter-regulator of glucocorticoid action [5,11,12]. A possible mode of action of MIF function is suggested by the surprising finding that MIF has enzymatic activity, showing both tautomerase [13,14] and thiol-protein oxidoreductase activity [15,16]. Adding further to an enzymatic role of MIF with likely physiological relevance has been the current failure to identify a conventional cytokine membrane receptor for MIF. A combined function both as a cytokine and as an enzyme would not be unique to MIF and has been shown previously for the protein mediators thioredoxin/adult T cell leukemia-derived factor (ADF) [17] or the cyclosporin A-binding factor cyclophilin [18].

Thiol-protein oxidoreductase activity has been investigated widely for proteins of the thioredoxin superfamily [19–22] and activity studies have been routinely performed using the insulin reduction assay [23,24]. MIF was recently shown to catalyze the reduction of insulin disulfides [15]. Further studies are needed to relate the identified enzymatic activity to the physiological activities of MIF. An important step towards this aim was taken in the present report. We studied the reduction of insulin disulfides by MIF using various physiologically occurring reductants, namely dihydrolipoamide, L-cysteine, and glutathione (GSH), as well as the non-natural substrates dithiothreitol (DTT) and β-mercaptoethanol. To confirm the catalytic role of the putative disulfide motif, Cys^{57}-Ala-Leu-Cys^{60} (CALC), of MIF for the insulin-reducing effect, reduction of insulin disulfides by wild-type human MIF (wtMIF) was compared to the effect brought about by the isosteric cysteine mutants C57S, C60S, C57S/C60S, and C81S MIF. Furthermore, the recently characterized carboxy-truncated mutants MIF(1–105) and MIF(1–110) with deletions at a distance from the putative catalytic center as well as trifluoroacetic acid (TFA)-modified MIF were examined. Lastly, induction of MIF expression by the oxidant hydrogen peroxide was analyzed by immunoblotting.

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. *Escherichia coli* thioredoxin (Trx) was bought from Calbiochem (Heidelberg, Germany). Glutathione, L-cysteine, DTT, and β-mercaptoethanol were from Sigma-Aldrich Chemicals. Dihydrolipoamide was a kind gift from F. Vitzthum (University of Stuttgart, Germany). Molecular biology reagents including oligonucleotide primers for PCR cloning were acquired from Gibco-BRL (Eggenstein, Germany) or New England Biolabs GmbH (Heidelberg, Germany).

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Abbreviations: MIF, macrophage migration inhibitory factor; GSH, reduced glutathione; DTT, dithiothreitol; wt, wild-type; TFA, trifluoroacetic acid

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PII: S0014-5793(98)00654-1

2.2. Site-directed mutagenesis, cloning, bacterial expression, and purification of wtMIF and the mutants

Human MIF was amplified originally from Jurkat H33HJ-JA1 T cell DNA and cloned into the pET11b expression vector [6]. The isosteric Cys→Ser mutants and the C-terminal deletion mutants of human MIF were cloned from huMIF/pET11b by DNA amplification as described [15,16].

Bacterial expression and purification of wtMIF and the mutants are described in detail elsewhere [6,15,16] (Kleemann et al., submitted). Briefly, wtMIF, the Cys→Ser mutants (except for mutants C60S and C57S/C60S), and the carboxy-truncated mutants were expressed and purified essentially following the established protocol for wtMIF [6], using isopropyl-1-thio-β-D-galactopyranoside-induced overexpression of proteins in pET11b/*E. coli* BL21(DE3) and purification of recombinant protein by the anion exchange and C8-SepPak reverse-phase two-step chromatography procedure, and renaturation of acetonitrile-denatured protein by controlled dialysis. Purification of mutants C60S and C57S/C60S was performed by a separate method involving extraction of the protein from inclusion bodies, gel filtration, and C8 reverse-phase chromatography (Kleemann et al., submitted).

2.3. Insulin reduction assay

MIF-catalyzed insulin reduction activity was examined following the procedure of Chandler and Varandani [23] and Holmgren [24]. Briefly, the insulin assay is based on the reduction of insulin and subsequent insolubilization of the insulin β-chain. The time-dependent increase in turbidity is then measured spectrophotometrically at 650 nm. Under standard conditions, the reaction was started by adding 177.5 μl of renatured MIF, TFA-modified MIF, or the mutants (dissolved in 20 mM sodium phosphate buffer, pH 7.2) or a control solution (containing buffer alone), and 22.5 μl reductant to 700 μl of the ice-cold reaction mixture containing 1 mg/ml insulin, 100 mM sodium phosphate buffer, pH 7.2, and 2 mM EDTA. The final concentration of wtMIF, TFA-modified MIF, and the MIF mutants was 1.8 μM. MIF-catalyzed insulin reduction was measured against the control solution in the same experiment. For measuring TFA-modified MIF, 0.005% TFA was added to the control solution. Samples were gently inverted every 10 min and turbidity followed for at least 2 h.

Mutant C57S/C60S was renatured by shock dilution from a 100% (v/v) 2,2,2,2′,2′,2′-hexafluoroisopropanol (HFIP) stock solution (Kleemann et al., submitted). The final concentration of HFIP in these samples was 0.2% (v/v). Control reactions for this mutant containing wtMIF were renatured by an identical procedure and therefore also contained 0.2% HFIP.

For analysis of the various substrates, an essentially identical protocol was applied. The reductants, except for dihydrolipoamide, were dissolved in water and added to the assay at various concentrations. Dihydrolipoamide was dissolved in acetone to prevent spontaneous oxidation and 2.25 μl of this solution were added to the assay to yield a final concentration of 0.6 mM [24]. Prior experiments had demonstrated that small volumes of up to 5 μl acetone did not interfere with the test.

The rate of precipitation of the insulin β-chain and the time of onset were determined as described previously [15,24]. Data (rate of precipitation) for the substrate screen using wtMIF represent at least four independently performed experiments. Data representing the insulin-reducing activity (rate of precipitation) of the mutants were from at least five independent experiments and represent net curves with the control spectra subtracted.

2.4. Cell culture and hydrogen peroxide induction

COS-1 cells were obtained from the German collection of microorganisms and cell lines (DSMZ, Braunschweig, Germany) and RAW 264.7 cells were a kind donation from R. Bucala (The Picower Institute for Medical Research, Manhasset, NY). COS-1 cells were maintained in EMEM medium (Gibco-BRL, Eggenstein, Germany) containing 10% heat-inactivated fetal calf serum (FCS). RAW 264.7 cells were cultured in RPMI 1640 medium containing 10% FCS. For induction experiments, cells were transferred into 3.5-cm tissue culture plates (2×10^6 cells/well), washed in the respective media containing 1% heat-inactivated FCS, and incubated in these media in a humidified atmosphere (37°C and 5% CO_2) for 1 h prior to H_2O_2 induction. We first confirmed earlier findings that addition of 0.2 mM H_2O_2 did not cause measurable cytotoxicity [25]. For induction, cells were treated stepwise with 0.01 mM H_2O_2 for 3 h and then with 0.2 mM H_2O_2 for an additional 2.5 h. Culture supernatants were collected, concentrated 25-fold by membrane filtration (10 kDa cut-off) (Centricon-10; Amicon GmbH, Witten, Germany), concentrated supernatants and cell pellets boiled in Laemmli electrophoresis buffer, and proteins separated in 18% SDS polyacrylamide gels followed by Western blotting as described previously [4]. Blotting membranes were incubated first with polyclonal rabbit anti-mouse MIF serum and then with alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (Pierce-KMF Laborchemie, St. Augustin, Germany) (each diluted 1:1000). MIF was visualized with BCIP and NBT according to the manufacturer's protocol (Boehringer Mannheim, Mannheim, Germany).

3. Results and discussion

We recently found that MIF functions to catalyze the reduction of insulin and 2-hydroxyethyl disulfide (HED), thus exhibiting thiol-protein oxidoreductase activity [15]. To further investigate this novel function of MIF and to examine the physiological relevance of this enzymatic activity, we tested several reducing substrates in the insulin reduction assay. This substrate screen revealed that only GSH and dihydrolipoamide were utilized by MIF (Fig. 1). By contrast, no significant reduction of insulin disulfides was observed when the reductants DTT, L-cysteine, and β-mercaptoethanol were used. Analysis of the rate of precipitation of insulin at various substrate concentrations showed that dihydrolipoamide was used best by MIF. MIF activity in the presence of this reductant was more than 10 times higher than MIF-catalyzed reduction of insulin by glutathione at a comparable substrate

Table 1
Catalysis of insulin reduction by wtMIF using various reductants

Substrate	Concentration [mM]	Rate of precipitation [A_{650}/h][a]	Time of onset [min]
Dihydrolipoamide	0.6	0.395	26 (35)[b]
Glutathione	1.0	0.033	197 (315)
	2.5	0.074	110 (185)
	5.0	0.189	60 (105)
L-Cysteine	1.0	0	145 (135)
	2.5	0	89 (89)
	5.0	0	57 (57)
β-Mercaptoethanol	1.0	0	160 (160)
	2.5	0.006	82 (82)
	5.0	0.035	50 (53)
Dithiothreitol	1.0	0	32 (32)
	5.0	0	13 (13)

Experiments were performed over a range of reductant concentrations and at a final MIF concentration of 1.8 μM.
[a]Data represent net rates of precipitation with the substrate control values subtracted.
[b]Numbers in parentheses represent the time of onset values of the control substrate reactions without enzyme.

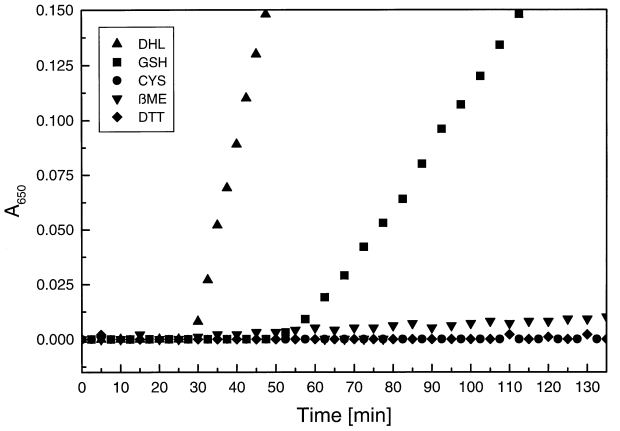

Fig. 1. MIF-catalyzed reduction of insulin by dihydrolipoamide (DHL), glutathione (GSH), L-cysteine (CYS), β-mercaptoethanol (βME), and dithiothreitol (DTT). Plot of the absorbance at 650 nm against time. Recombinant wtMIF was tested at a protein concentration of 1.8 μM with the respective reductants.

concentration (Table 1). Reduction by dihydrolipoamide may be even higher as the concentration of dihydrolipoamide used was limited by the concentration of acetone which was used to stably dissolve dihydrolipoamide and the dihydrolipoamide-dependent reaction was even faster than that mediated by GSH using 5 mM GSH (optimal concentration for GSH).

These findings were confirmed by the corresponding times that were necessary for the MIF-catalyzed reaction to set in (Table 1, time of onset). Reactions with dihydrolipoamide and GSH (5 mM) precipitated after 26 and 60 min, respectively, and were significantly faster than the substrate controls. By contrast, the reactions with DTT, L-cysteine, and β-mercaptoethanol precipitated considerably more slowly with values close to those of the corresponding control reactions without enzyme (Table 1).

In conclusion, specific utilization of GSH and dihydrolipoamide by MIF suggested that these substrates might represent physiological substrates of the investigated activity of MIF.

Oxidoreductase activity of MIF has been suggested to be dependent on the presence of the CALC motif in the center of the molecule [15]. Moreover, MIF mutants with the CALC-derived cysteines exchanged have been found to lack the macrophage-activating effects of MIF [15]. CXXC-motif-containing enzymes play a broad role in the regulation of cellular oxidation/reduction processes and the consensus stretch cysteines of these proteins are inter-converted between their dithiol and disulfide forms during the enzymatic reaction. Examples are thioredoxin, glutaredoxin, thioredoxin reductase, glutaredoxin reductase, or dihydrolipoamide dehydrogenase. To confirm whether the observed enzymatic conversion by MIF was mediated by the cysteines of the CXXC motif, i.e. Cys^{57} and Cys^{60}, and thus to further confirm the physiological relevance of the enzymatic activity of MIF, various isosteric mutants (Fig. 2) were subjected to the insulin reduction assay. Both single mutants with Cys^{57} or Cys^{60} substituted and the double mutant C57S/C60S were investigated. For control, mutant C81S with the third cysteine residue of MIF that is at distance from the CALC motif was used. In addition, two carboxy-truncated mutants of MIF, MIF(1–105) and MIF(1–110), were applied in the insulin reduction assay, as these mutants had previously been shown to exhibit enzymatic oxidoreductase activity while lacking the cytokine-like activities of MIF [16]. The C-terminal mutants have the C-terminal β-strands β6 and β7 deleted which have been proposed to play a role in trimer formation [16,26] of the MIF molecule, but

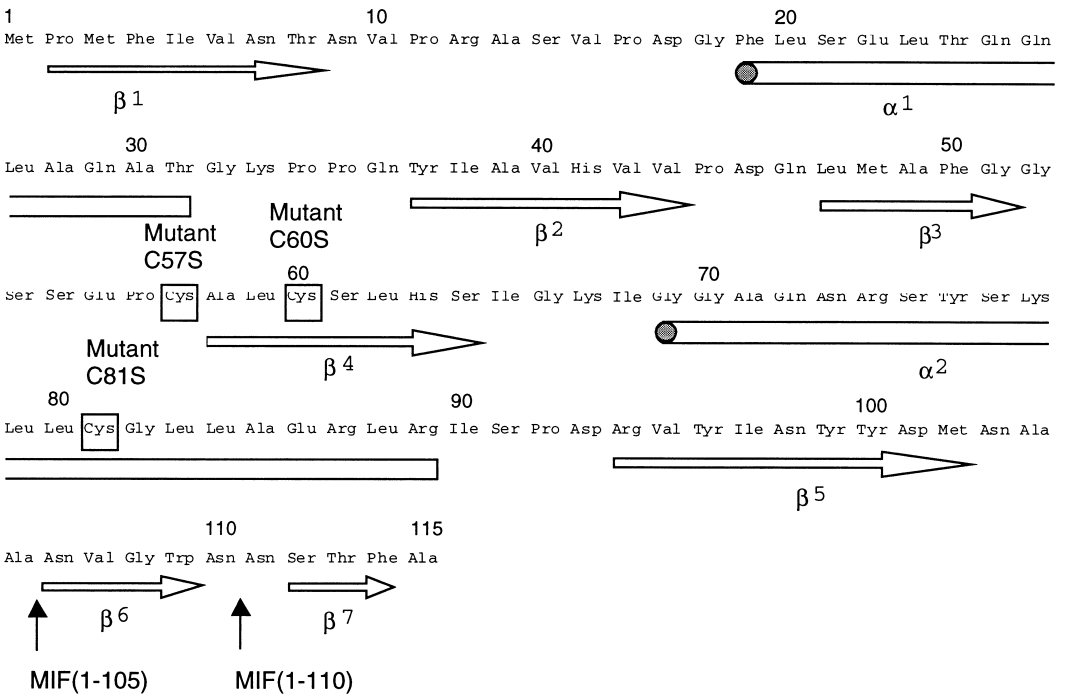

Fig. 2. Primary and secondary structure of human MIF and location of mutations. Open arrows indicate β-strands and cylinders represent α-helices. The sites of the deletions of mutant MIF(1–110) and MIF(1–105) are indicated by closed arrows and the sites of point mutations of the cysteine mutants C57S, C60S, C57S/C60S and C81S are indicated by squares. Of note, numbering of the amino acids refers to the cDNA sequence starting at Met^{1}.

deletions are at distance from the CALC motif by both sequence and three-dimensional means (Fig. 2).

Mutants were tested using the physiological substrate GSH and the reactions compared to wtMIF (Fig. 3A,B). Insulin precipitation curves showed that mutant C60S was essentially inactive. This mutant only showed 14 ± 5% activity when compared to wtMIF. The rate of insulin reduction by mutant C57S was markedly faster than that by mutant C60S (51 ± 13% of the activity of wtMIF), but no significant difference was observed when the times of onset were compared for these two mutants. Mutant C81S exhibited significant activity (70 ± 12% of the wild-type control). The observed reduction in activity of mutant C81S compared to the wild type protein is likely to represent a conformational effect rather than to indicate a direct contribution of Cys^{81} towards the enzymatic reaction, as mutant C81S exhibits an overall activity spectrum that is similar to wtMIF (Kleemann et al., in preparation). Mutant C57S/C60S which required 0.2% HFIP to be present in the reaction mixture was measured in a separate reaction.

Previous attempts to measure the enzymatic activity of this mutant had failed due to the delicate solubility behavior of this protein and the requirement to include HFIP in the solvents [15]. Here, we found that 0.2% (v/v) HFIP did not interfere with insulin reduction enabling us for the first time to analyze the biological activity of mutant C57S/C60S. Confirming the above data obtained for the single mutants C57S and C60S, mutant C57S/C60S was found to exhibit only 20 ± 3% of the activity of wtMIF (Fig. 3B).

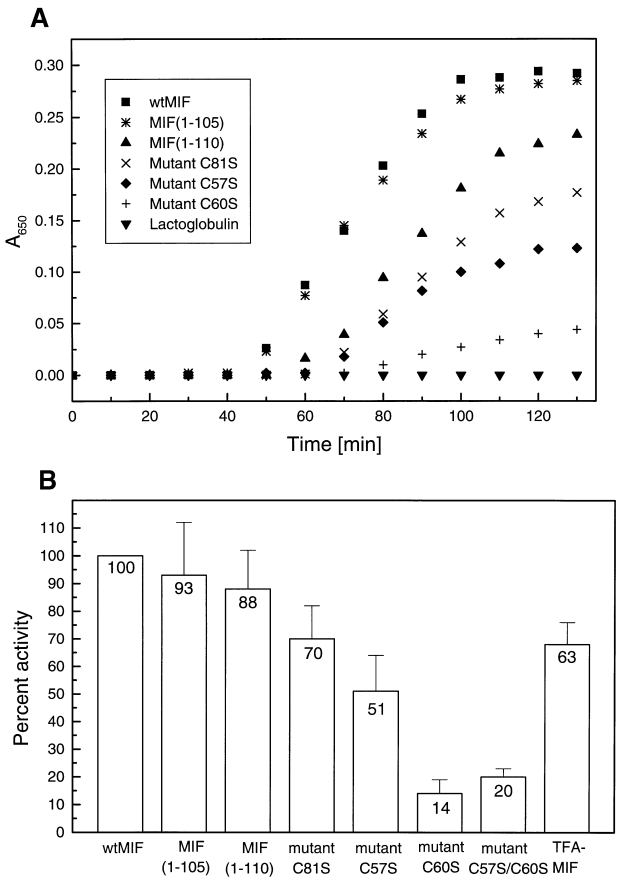

Fig. 3. Comparison of the various MIF mutants with wtMIF in the MIF-catalyzed reduction of insulin by GSH. A: Plot of the absorbance at 650 nm against time. Mutants were used at a protein concentration of 1.8 μM and compared to wtMIF at the same concentration. Lactoglobulin and 20 mM sodium phosphate buffer, pH 7.2 (NaPP) containing the concentration of GSH, were used as negative controls. For better comparison, curves represent net absorbances with substrate control values subtracted. Data are representative of three independent experiments. B: Relative activity of the mutants and TFA-modified MIF in comparison to the insulin-reducing effect of wtMIF. The numbers indicate the relative activities with wtMIF set at 100%. Data represent the mean ± S.E.M. of at least five assays.

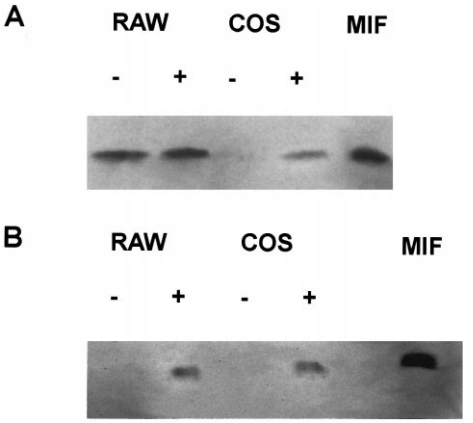

Fig. 4. Induction of MIF protein by the oxidant H_2O_2. Western blotting analysis of the intracellular (A) and secreted MIF levels (B) following incubation of RAW 264.7 and COS-1 cells in the presence (+) and absence (−) of H_2O_2. Recombinant human MIF (20 ng) was electrophoresed and transferred as a reference.

Analyses of the time of onset were in overall agreement with the measurements of the precipitation rate. Mutant C60S precipitated last, with measurable turbidity detected 17 min after wtMIF and close to the substrate control. Mutants C57S and C81S precipitated 6 min after the wild-type protein (data not shown). The double mutant C57S/C60S was tested in a separate reaction due to the special solvent requirements and thus the time of onset was not comparable to the other mutants. However, when compared to wtMIF tested under identical solvent conditions, this mutant precipitated 28 min slower than the wild-type protein and close to the substrate control (not shown).

Both C-terminal mutants exhibited significant insulin-reducing activity when compared to wtMIF. We found that the carboxy-truncated mutants exhibited an activity very similar to wtMIF (Fig. 3A). The rates of precipitation of insulin measured for these mutants were almost identical to that of wtMIF (Fig. 3B) and the times of onset were closest to the wild-type protein among all mutants (not shown). As expected, this demonstrated that the C-terminus of MIF was not involved in the reduction of insulin disulfides.

The obtained data, in particular the successful analysis of mutant C57S/C60S requiring organic solvent to be present, indicated to us that the insulin reduction assay could be a valuable tool for structure activity investigations using a broader range of MIF mutants and chemically modified MIF derivatives. As a first step towards this aim, we applied TFA-modified wtMIF to the assay. TFA is widely known to react with basic amino acids such as Lys, Arg, and His to form the corresponding TFA salts. TFA modification of MIF was performed by incubating wtMIF in 0.005% (v/v) TFA for 60 min. The resulting TFA-modified MIF showed normal solubility and was subjected to the insulin reduction

assay without further purification. TFA-modified MIF exhibited 63 ± 8% of the activity of wtMIF (Fig. 3B) and catalysis started 12 min later than that by wtMIF (not shown). Thus, TFA-modified MIF that behaved normally otherwise was only partially active. However, activity of this derivative was much higher than the activities of the essentially inactive mutants C60S and C57S/C60S. It is likely, therefore, that modification of basic amino acids led to a number of small conformational changes resulting in the observed reduced activity compared to wtMIF. Interestingly, modification of MIF by fatty acids has also been reported to result in a reduced activity of MIF [27] and such a modification is likely to also involve basic amino acids.

In summary, these results confirmed the role of the CALC sequence for the oxidoreductase activity of MIF. In particular, the importance of Cys^{60} which was found previously to be essential for the HED-reducing and macrophage-activating activities of MIF [15] was corroborated. Moreover, the finding that the cysteines of the CXXC motif were involved in conjunction with the measured specificity for the substrates GSH and dihydrolipoamide suggested that MIF may play a role in the regulation of the cellular redox homeostasis. Glutathione is a potent sulfhydryl reductant and plays a key role in maintaining the cellular redox balance [28]. In this function, GSH serves to protect cellular function against oxidative stress and apoptosis [29]. Dihydrolipoamide has been shown to serve as a therapeutic antioxidant with vitamin E- and GSH-like antioxidant activity [30–33]. The redox potentials of the dihydrolipoamide/lipoamide and GSH/GSSG couple are −0.29 and −0.25 V, respectively [34]. By comparison, the redox potential for the oxidized form of thioredoxin, i.e. the classical insulin-reducing protein, is −0.26 V [24]. A redox potential for MIF has not yet been determined, but the current data would suggest that MIF also exhibits a reducing redox potential. One putative MIF-regulated target within the cell is suggested by the MIF-mediated antagonism of the immunosuppressive effects of glucocorticoids [5]. The glucocorticoid receptor which has been thought to be regulated in its steroid-binding activity by oxidation/reduction of a vicinally spaced pair of cysteine residues [35] could be inactivated by a MIF-mediated redox event in which GSH and/or dihydrolipoamide may be the corresponding reductants.

Oxidants such as H_2O_2 have been shown to impose oxidative stress onto cells leading to oxidation of the cellular GSH pool [36]. To begin to investigate the role of MIF within the complex cellular redox network, for example during oxidative stress, induction of MIF by COS-1 and RAW 264.7 cells was examined following treatment with H_2O_2. Treatment of both cell lines with 0.2 mM H_2O_2 and subsequent Western blotting analysis with a polyclonal anti-MIF antibody [4] resulted in a marked secretion of MIF into the culture media, whereas no increase in secretion was seen in the untreated control cells. For COS-1 cells, which express only low levels of MIF protein at baseline (A. Braun, unpublished observations), induction by H_2O_2 also led to a significant increase in the intracellular levels of MIF. Only a small H_2O_2-mediated increase in intracellular MIF was observed in the RAW 264.7 macrophage (Fig. 4). RAW 264.7 cells contain high baseline levels of intracellular MIF that are released upon immune stimulation. Although intracellular induction of MIF protein, i.e. de novo synthesis, has not been studied in this cell type, immunological induction of MIF mRNA levels has been shown to parallel secretion of MIF protein. Thus, it would appear likely that the H_2O_2-induced, intracellular levels of MIF were too small to be detected, but that intracellular induction was clearly indicated by the observed induction of MIF secretion. Overall, these data were in accordance with a suggested role for MIF in oxidative stress and cellular redox processes. By contrast, the recent identification of MIF in the pancreas and its colocalization to the insulin secretory granules of the pancreatic islet β-cell [37] is probably incidental and unlikely to indicate a role for MIF in the regulation of insulin secretion via the reduction of the insulin disulfides.

Acknowledgements: We thank F. Vitzthum, R. Bucala, and T. Calandra for helpful discussions. J.B. is supported by the Deutsche Forschungsgemeinschaft (DFG) Grant BE 1977/1-1. We are grateful to F. Vitzthum for providing the dihydrolipoamide.

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