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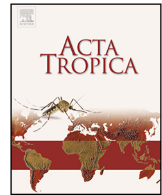

Role of cysteine-58 and cysteine-95 residues in the thiol di-sulfide oxidoreductase activity of Macrophage Migration Inhibitory Factor-2 of *Wuchereria bancrofti*

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ABSTRACT

Macrophage Migration Inhibitory Factor (MIF) is the first human cytokine reported and was thought to have a central role in the regulation of inflammatory responses. Homologs of this molecule have been reported in bacteria, invertebrates and plants. Apart from cytokine activity, it also has two catalytic activities viz., tautomerase and di-sulfide oxidoreductase, which appear to be involved in immunological functions. The CXXC catalytic site is responsible for di-sulfide oxidoreductase activity of MIF. We have recently reported thiol-disulfide oxidoreductase activity of Macrophage Migration Inhibitory Factor-2 of *Wuchereria bancrofti* (Wba-MIF-2), although it lacks the CXXC motif. We hypothesized that three conserved cysteine residues might be involved in the formation of di-sulfide oxidoreductase catalytic site. Homology modeling of Wba-MIF-2 showed that among the three cysteine residues, Cys_{58} and Cys_{95} residues came in close proximity (3.23 Å) in the tertiary structure with pK_a value 9, indicating that these residues might play a role in the di-sulfide oxidoreductase catalytic activity. We carried out site directed mutagenesis of these residues (Cys_{58}Ser & Cys_{95}Ser) and expressed mutant proteins in *Escherichia coli*. The mutant proteins did not show any oxidoreductase activity in the insulin reduction assay, thus indicating that these two cysteine residues are vital for the catalytic activity of Wba-MIF-2.
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1. Introduction

Macrophage Migration Inhibitory Factor (MIF) is the first cytokine to be described from human in late 1960s. Human MIF (hMIF) is secreted by several types of cells such as macrophages, eosinophills, β-cells of the islets of Langerhans, keratinocytes and T-cells (Prieto-Lafuente et al., 2009). It is an inflammatory mediator with the ability to induce immunomodulatory responses through Th1 type response and overrides glucocorticoid-driven immune suppression (Kudrin et al., 2006). It is involved both in the innate and adaptive immunity and stimulates the production of matrix metalloproteases, cyclooxygenase2 and prostaglandin E2 (Leng et al., 2003; Mitchell et al., 2002). It activates and enhances the expression of several pro-inflammatory cytokines such as TNF-α, IL1, IL2, IL8, and IFN-γ (Bacher et al., 1996; Donnelly and Bucala, 1997; Calandra et al., 2000). It is also a ligand for the CD74–CD44 receptor complex present at the surface of target cells. It induces the expression of these receptors leading to the modulation of cytokine expression and counter-regulation of the anti-inflammatory and immunosuppressive effects of glucocorticoid steroids (Flaster et al., 2007). Presence of MIF is reported to be essential for resistance against pathogens as MIF deficient mice succumbed to low doses of *Salmonella typhimurium* infection and show increased susceptibility to parasitic protozoans (*Leishmania major* and *Toxoplasma gondii*), and cestode (*Taenia crassiceps*) (Koebernick et al., 2002; Satoskar et al., 2001; Flores et al., 2008). Recently, the hMIF has generated a lot of interest as it is found to be involved in the pathogenesis of inflammatory disorders such as rheumatoid arthritis, atherosclerosis, diabetes, sepsis and inflammatory bowel diseases (Lue et al., 2002).

Homologs of hMIF have been reported from several parasite species belonging to both protozoans and nematodes. Among nematodes, MIF homologs have been reported from free living nematode *Caenorhabditis elegans* and parasitic worms such as *Trichuris* sp., *Onchocerca volvulus*, *Brugia malayi*, *Brugia pahangi*, *Strongyloides ratti*, *Haemonchus contortus*, *Ancylostoma* sp., and

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Schistosoma sp. (Pastrana et al., 1998; Tan et al., 2001; Zang et al., 2002; Wu et al., 2009; Miska et al., 2007; Augustijn et al., 2007; Kamir et al., 2008; Vermeire et al., 2008; Younis et al., 2012; Ajonina-Ekoti et al., 2013). The lymphatic filarial parasite, *B. malayi* produces two homologs of MIF, Bma-MIF-1 and Bma-MIF-2, with 40% and 27% identity with the hMIF, respectively (Zang et al., 2002). Parasite MIF activates macrophages to an alternative phenotype that contributes to counter-inflammation during *Brugia* infection (Prieto-Lafuente et al., 2009). Several studies have shown that parasite-derived MIF modulates immunity of the host such that the parasite survival is enhanced. Recently, interest has been elicited on the use of antigenic molecules of parasite origin as therapeutic agents for the treatment of inflammatory diseases, and MIF-2 from intestinal parasite *Anisakis simplex* has been shown to ameliorate intestinal colitis (Cho et al., 2011). Interest has also been generated to explore this parasite cytokine as a drug target for developing inhibitors that prevent entry or expel parasites from host (Dios et al., 2002; Crichlow et al., 2007).

Earlier, we have reported the characterization of MIF-1 (Wba-mif-1) and MIF-2 (Wba-mif-2) from *Wuchereria bancrofti* (Sharma et al., 2012; Chauhan et al., 2015), the major causal organism of human lymphatic filariasis. The alignment of derived amino acid sequences of Wba-MIF-2 with Wba-MIF-1 showed 44% homology. The pleotropic hMIF, apart from having cytokine functions, also has two enzymatic activities viz., tautomerase and oxidoreductase activities (Rosengren et al., 1996; Bendrat et al., 1997; Swope et al., 1998) by virtue of which it counteracts the glucocorticoid actions (Lubetsky et al., 2002). Similar to hMIF the Bma-MIF-1 also possesses two enzymatic activities viz., tautomerase and oxidoreductase, and conserved proline residue at N-terminal end and CXXC motif are essential for the two activities, respectively (Pastrana et al., 1998). Presumably, Bma-MIF-2 lacks the oxidoreductase activity unlike MIF-1, as it does not have CXXC motif (Zang et al., 2002). Wba-MIF-2 also lacks the CXXC motif but, we found significant oxidoreductase activity of this homolog in the insulin reduction assay (Chauhan et al., 2015), plausibly because of the presence of other vicinal cysteine residues. Two conserved vicinal cysteine residues of MIF are reported to play a role in the oxidoreductase activity, because of oxidation and reduction properties of SH- groups. In *Plasmodium falciparum* MIF (Alam et al., 2011), the oxidoreductase activity is reported to be due to two conserved vicinal cysteine residues (Cys_3 and Cys_4) at N-terminal, in the absence of CXXC motif. In the present study, since the CXXC motif is absent in Wba-MIF-2 we examined the role of cysteine residues in its oxidoreductase activity through homology modeling and site directed mutagenesis and the results are presented here.

2. Materials and methods

2.1. Sequence alignment of MIF homologs

The sequences of MIF homologs reported in different organisms were obtained from the GenBank database. The derived amino acid sequence from Wba-mif-2 mRNA sequence (GenBank accession number—KJ939449) was aligned with amino acid sequences of other known MIF homologs using ClustalW algorithm in BioEdit sequence alignment editor (Hall, 1999). The position of conserved cysteine residues were determined from aligned sequences.

2.2. Homology modeling of Wba-MIF-2

The tertiary structure of Wba-MIF-2 was obtained by using 'Protein Homology/Analogy Recognition Engine' (PHYRE2) (Kelley and Sternberg, 2009). The Ramachandran plot of Wba-MIF-2 modeled protein was analyzed in PROCHECK for allowed region of the amino acids placed in Wba-MIF-2 modeled structure (Laskowski et al., 1993). Further validation of the modeled 3D structure of Wba-MIF-2 was performed in ProQ server (Cristobal et al., 2001). The location of all the three conserved cysteine residues was traced in the modeled structure and assessed for their proximity to each other. The cysteine residues surrounding amino acids were identified within a range of 5 Å. The pK_a values of cysteine residues were calculated using Discovery studio 4.1 visualizer.

2.3. Site directed mutagenesis

Site-directed mutagenesis was carried out by primer extension method (Ho et al., 1989) incorporating mutagenized primers in independent PCRs, and the resulting fragments were ligated to form complete Wba-MIF-2 cDNA sequence. For achieving this, primers for specific mutants were designed within a range of same Tm to amplify at same PCR cycle condition and for ease in their ligation in consequent assembly in separate PCR reaction. Proof reading DNA polymerase enzyme (Merck, India) was used in the PCR to reduce the chance of any secondary mutation. The PCR cycle condition was as follows: initial denaturation at 94 °C for 4 min with 30 cycles of denaturation at 94 °C for 20 s, annealing at 55 °C for 30 s, extension at 72 °C for 45 s, with final extension at 72 °C for 10 min. During the first round of PCR, the AB and CF fragments of length 183 bp and 180 bp of mutant Cys_{58}Ser were created and in another PCR, AD and EF fragments of length 288 bp and 75 bp of mutant Cys_{95}Ser were produced. These amplicons of individual mutant were mixed and used for the second round of PCR as template employing primers A and F at same PCR cycle parameters described above.

2.4. Cloning and expression of the Wba-MIF-2 mutants

The assembled coding sequences of Wba-MIF-2 were subjected to sequencing for confirmation of mutation created at specific positions. The amplified mutated PCR products were treated with restriction enzymes PstI and Hind III (New England Biolabs, USA) in double digestion for 3 h at 37 °C and used in the ligation reaction with expression vector pRSETB using Quick ligation kit (New England Biolabs, USA) as per recommended protocol. Ligation mixtures carrying the mutated inserts were used to transform *Escherichia coli* DH5α, plated on to LB plates supplemented with 100 μg/ml ampicillin and incubated at 37 °C for overnight. The clones which appeared were grown in LB medium supplemented with 100 μg/ml ampicillin and the plasmid constructs were isolated using Nucleospin plasmid kit (Macherey-Nagel, Germany). The construct from the positive clones of both mutants were checked for the presence of open reading frame (ORF) by sequencing. The mutated constructs were transformed into *E. coli* strain GJ1158 for expression of the desired mutated proteins carrying serine instead of cysteine. Positive GJ1158 clones were grown overnight and inoculated to one liter of fresh LBON medium at 0.5% level. After the culture reached optimal $O.D._{600}$ of 0.6, it was induced with 300 mM sterilized NaCl for 3 h. The culture broth was centrifuged at 12,000 rpm for 10 min and the resulting pellet was re-suspended and sonicated in lysis buffer containing 50 mM Tris, 50 mM of Na_2HPO_4, 400 mM NaCl and 0.3 mg/ml lysozyme. The sonicated pellet was centrifuged at 14,000 rpm for 20 min at 4 °C to recover the clear supernatant, in a high speed centrifuge (Hitachi, Japan).

2.5. Purification of recombinant Wba-MIF-2 protein

The recovered supernatant of whole cell lysate of un-induced culture and induced culture of *E. coli* GJ1158 was checked for the expression of mutated recombinant protein in 12% SDS PAGE (Laemmli, 1970). The mutated proteins expressed as histidine tag fusion proteins, were purified by immobilized metal affinity

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