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MIF proteins are theta-class glutathione *S*-transferase homologs

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Abstract

MIF proteins are mammalian polypeptides of approximately 13,000 molecular weight. This class includes human macrophage migration inhibitory factor (MIF), a rat liver protein that has glutathione *S*-transferase (GST) activity (TRANSMIF), and the mouse delayed early response gene 6 (DER6) protein. MIF proteins were previously linked to GSTs by demonstrating transferase activity and observing N-terminal sequence homology with a mu-class GST (Blocki, F.A., Schlievert, P.M., & Wackett, L.P., 1992, *Nature 360*, 269-270). In this study, MIF proteins are shown to be structurally related to the theta class of GSTs. This is established in three ways. First, unique primary sequence patterns are developed for each of the GST gene classes. The patterns identify the three MIF proteins as theta-like transferase homologs. Second, pattern analysis indicates that GST members of the theta class contain a serine residue in place of the N-terminal tyrosine that is implicated in glutathione deprotonation and activation in GSTs of known structure (Liu, S., et al., 1992, *J. Biol. Chem. 267*, 4296-4299). The MIF proteins contain a threonine at this position. Third, polyclonal antibodies raised against recombinant human MIF cross-react on Western blots with rat theta GST but not with alpha and mu GSTs. That MIF proteins have glutathione-binding ability may provide a common structural key toward understanding the varied functions of this widely distributed emerging gene family. Because theta is thought to be the most ancient evolutionary GST class, MIF proteins may have diverged early in evolution but retained a glutathione-binding domain.

Keywords: DM4 dichloromethane dehalogenase; glutathione activation; glutathione *S*-transferase; inhibitory factor; macrophage migration

Over 50 dimeric cytosolic glutathione *S*-transferases from more than 20 species comprise a family of detoxification enzymes able to conjugate glutathione (GSH: γ-L-glutamyl-L-cysteinyl-glycine) to a wide range of compounds (Boyer & Kenney, 1985; Warholm et al., 1985; Armstrong, 1991). Four GST gene classes, based on subunit primary structure, are recognized: alpha, mu, pi, and theta (Mannervik & Danielson, 1988; Meyer et al., 1991; Ogura et al., 1991). Crystal structures of pi (Reinemer et al., 1991, 1992) and mu (Ji et al., 1992), as well as site-directed mutagenesis of alpha (Stenberg et al., 1991) and pi (Kolm et al., 1992; Manoharan et al., 1992) GSTs have established the essential role of a conserved N-terminal active-site tyrosine residue. This tyrosine's hydroxyl group is implicated in the deprotonation of the glutathione thiol group to generate a more reactive thiolate nucleophile (Liu et al., 1992). Sequence analysis and biological distribution suggest that the theta GSTs may be the progenitors from which the other GSTs differentiated (Pemble & Taylor, 1992).

A class of small mammalian proteins resembling human macrophage migration inhibitory factor were recently shown to be related to GSTs (Blocki et al., 1992). The MIF proteins from rat liver (now called TRANSMIF [Blocki et al., 1992]), human T-cell supernatants (Weiser et al., 1989; Tagaya et al., 1993), pituitary (Bernhagen et al., 1993), and embryonic eye lens (Wistow et al., 1993) are approximately 13,000 molecular weight and have extensive sequence homology with each other. Human MIF is a lymphokine involved in delayed hypersensitivity (Rocklin et al., 1970). The precise biological functions

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Abbreviations: GenBank, NIH nucleic acid sequence data bank; PIR, Protein Identification Resource data bank; Swiss-Prot, University of Geneva protein sequence data bank; EMBL, European Molecular Biology Laboratory; PIMA, pattern-induced multisequence alignment; GST, glutathione *S*-transferase; MIF, macrophage migration inhibitory factor; DCMD DM4, bacterial dichloromethane dehalogenase; DER6, delayed early response protein 6; GIF, glycosylation inhibiting factor; NO, nitric oxide.

of the other MIF proteins are less defined. TRANSMIF was the first member of the class with demonstrated GST activity and modest observed N-terminal sequence homology to a mu GST (Blocki et al., 1992).

Here we report computational and experimental evidence that the MIF-like proteins are homologs of theta-class GSTs. First, class-specific GST N-terminal primary sequence patterns were developed. The theta-class pattern uniquely identified all seven known theta GSTs and MIF proteins of the 40,298 entries in the PIR32 data bank. Second, theta-class GSTs have serine in a comparable position to the binding-site tyrosine found in the alpha, mu, and pi GST classes. MIF proteins contain threonine in this position. Third, anti-MIF antisera cross-react with two previously established theta-class GSTs, but not with alpha and mu GSTs.

Results

Class-specific patterns

Sequences of 35 N-terminal amino acid residues from all 54 known GST superfamily members (Table 1) were aligned (Smith & Smith, 1990, 1992) (Fig. 1). Protein pairs with the highest similarity scores (joined lower on the ordinate) are most similar. Consistent with the work by others, members of the GST superfamily segregated into four distinct clusters: alpha, mu, pi, and theta. However, new to this work, a distinct cluster of six plant loci, labeled "phi," also appears and is more closely related to the alpha–pi–mu GSTs than are the 10 theta-class members. Previous work has classed the phi GSTs in the theta class (Pemble & Taylor, 1992). TRANSMIF, human MIF, and DER6 form a tight theta subcluster that is more closely related to the other seven theta-class members than to the six phi GSTs (see Fig. 1).

The residue positions used in our class-specific patterns were the most highly conserved positions (i.e., contained the least number of amino acid alternatives) across the whole GST superfamily. Eleven such positions were needed, as described later, to produce 100% specific GST patterns. Positions other than these 11 may be more strongly conserved within a given GST gene class. However, by being most strongly conserved across the entire GST superfamily, these 11 positions provide the best descriptors of the common N-terminal structural motif involved in glutathione binding.

As shown in Table 2, the 11 positions are 3, 5, 7, 10, 11, 12, 16, 18, 21, 24, and 28, based on the residue numbers of the pi GST (Reinemer et al., 1991). These residue

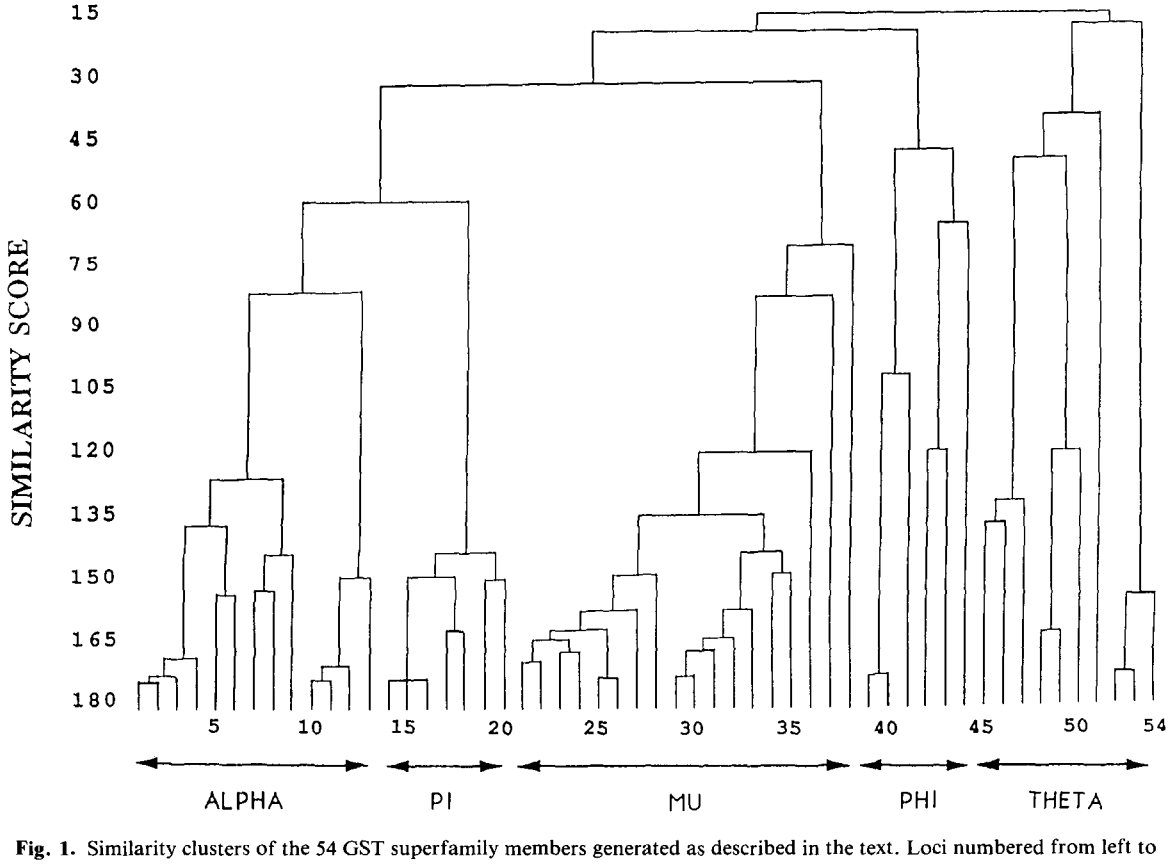

Fig. 1. Similarity clusters of the 54 GST superfamily members generated as described in the text. Loci numbered from left to right are in the order of appearance depicted in Table 1. Higher similarity scores indicate greater relatedness.

Table 1. *PIR32 data base glutathione S-transferases in order of similarity and segregated into classes*

PIR locus[a]	Common name	Species	Order	Class grouping
A27848	GST Ya	*Mus musculus*	1	Alpha
C28946	GST alpha	*Mus musculus*	2	
XURTG	GST Ya	*Rattus norvegicus*	3	
A26653	GST Ya	*Rattus norvegicus*	4	
A25909	GST 2	*Homo sapiens*	5	
A29723	GST 2	*Homo sapiens*	6	
A26753	GST Yc	*Rattus norvegicus*	7	
CHKCL3[1]	GST	*Gallus gallus*	8	
XURT8C	GST 8	*Rattus norvegicus*	9	
Sb28GST[2]	GST	*Schistosoma bovis*	10	
Sh28GST[2]	GST	*Schistosoma haematobium*	11	
A26598	28K antigen precursor (GST)	*Schistosoma mansoni*	12	
Sj28GST[2]	GST	*Schistosoma japonicum*	13	
S03015	GST pi	*Homo sapiens*	14	Pi
S09316	GST pi	*Homo sapiens*	15	
S01672	GST pi	*Homo sapiens*	16	
A26546	GST P	*Rattus norvegicus*	17	
S12709	GST pi	*Mus musculus*	18	
S13780	GST pi	*Sus scrofa domestica*	19	
S16392	GST	*Bos primigenius taurus*	20	
A23732	GST mu	*Mesocricetus auratus*	21	Mu
B34159	GST mu 2	*Mus musculus*	22	
A29036	GST Yb3	*Rattus norvegicus*	23	
A39375	GST GST4	*Homo sapiens*	24	
A30770	GST mu	*Homo sapiens*	25	
S01719	GST 4	*Homo sapiens*	26	
B26187	GST Yb2	*Rattus norvegicus*	27	
A35295	GST mu	*Homo sapiens*	28	
A24085	GST Yb1	*Rattus norvegicus*	29	
A25510	GST Yb	*Rattus norvegicus*	30	
A28946	GST mu 8.7	*Mus musculus*	31	
S13202	Y1 protein (GST mu)	*Cricetulus griseus*	32	
B29231	GST Yb4	*Rattus norvegicus*	33	
B28946	GST mu 9.3	*Mus musculus*	34	
JX0095	GST b	*Cavia porcellus*	35	
S14636	GST CL2	*Gallus gallus*	36	
A26484	GST	*Schistosoma japonicum*	37	
S03615	GST P	*Caenorhabditis elegans*	38	
XUZM31	GST III	*Zea mays* (version 1)	39	Phi
XUZM32	GST III	*Zea mays* (version 2)	40	
XUZM1	GST I	*Zea mays*	41	
LVWORT[3]	GST	*Silene cucubalus*	42	
TBACCO[4]	GST par B	*Nicotiana tabacum*	43	
S16604	GST	*Dianthus caryophyllus*	44	
JS0618	GST Yrs	*Rattus norvegicus*	45	Theta
S14346	GST 12	*Homo sapiens*	46	
S14345	GST 5	*Rattus norvegicus*	47	
S16293	GST	*Musca domestica*	48	
XUFF11	GST 1-1	*Drosophila melanogaster*	49	
JQ1378	GST 27	*Drosophila melanogaster*	50	
DCMD DM4[5]	Dichloromethane dehalogenase	*Methylobacterium* sp.	51	
DER6[6]	Delayed factor-induced early response gene 6	*Mus musculus*	52	
S27117	TRANSMIF	*Rattus norvegicus*	53	
A33838	Macrophage migration inhibitory 12.7K protein	*Homo sapiens*	54	

[a] Loci not deposited in PIR32 at time of this investigation were retrieved from the literature: [1]Chang et al., 1992; [2]Trottein et al., 1992; [3]Prandl and Kutchan, 1992; [4]Takahashi and Nagata, 1992; [5]LaRoche and Leisinger, 1990; [6]Lanahan et al., 1992. These locus names were arbitrarily chosen.

Table 2. *Class-specific residues giving 100% discrimination for five GST classes*

Residue number[a]	Class				
	Theta	Phi	Alpha	Pi	Mu
3	FLY	AKPT	IPV	Y	LMPV
5	LPV	KQ	LV	IV	L
7	ST	HY	Y	Y	Y
10	CPS	PSV	AGI	V	ITV
11	R	LMR	R	QR	HKR
12	AS	S	G	G	G
16	AGM	R	CPSV	A	APS
18	AELK	LAR	R	R	R
21	ILV	L	L	L	L
24	ENQ	AK	A	Q	KLT
28	ALV	FY	FY	W	Y

[a] Residue position based on the pig lung pi GST crystal structure (Reinemer et al., 1991). Alpha, mu, and pi GST classes have a gap of three between residue position 12 and residue position 16; the theta and phi GST classes have a gap of four.

positions are superimposed upon a linearized ribbon diagram of the pi GSTs' first three secondary elements in Figure 2; together they compose the N-terminal structural motif identified in this study, which is diagnostic of the GST superfamily.

Alternative residues were iteratively tested at different positions until a collection was found that matched each of the four gene classes and the new phi cluster in the GST superfamily. These five class-specific GST sequence patterns are shown in Table 2. They distinguish between five GST classes with 100% sensitivity and 100% specificity.

For example, THETA, the theta-class-specific pattern, consists of the following 11 residue groupings in a 27-residue span:

[FLY] X [LPV] X **[ST]** XX [CPS] [R] [AS] XXXX
[AGM] X [AELK] XX [ILV] XX [ENQ] XXX [ALV]

where, for a given position, square brackets enclose residue alternatives, and X indicates any residue. THETA is found only in the 10 theta proteins (S16293, XUFF11, JQ1378, JS0618, S14345, S14346, human MIF [A33838], TRANSMIF [S27117], DER6, and DCMD DM4; see Table 1) of the set of 54 GST superfamily members *and* all 40,298 proteins in the PIR32 data bank.

The corresponding class-specific patterns ALPHA, MU, PI, and PHI, are found in only the 13 alpha, 18 mu, 7 pi, and 6 phi GSTs, respectively, of the 54 members of the GST superfamily and the 40,298 proteins in the PIR32 data bank.

Binding site tyrosine

The bold **[ST]** at position 7 of THETA indicates the serine (loci 45–51, Table 1) or threonine (loci 52–54) found in the theta GST class, in place of the otherwise universally conserved tyrosine found at residue numbers 6, 7, or 9 found in mu, pi, and alpha classes, respectively. Of the phi GSTs, those from liverwort (Prandl & Kutchan, 1992) and tobacco (Takahashi & Nagata, 1992) have histidine, whereas the remaining four have tyrosine (Table 2) at pattern position 7.

Western immunoblot analysis

The immune cross-reactivity of human MIF, TRANSMIF, DCMD DM4, and a partially purified sample of theta 5-5 GST from rat liver was studied as described in the Materials and methods. Theta 5 antisera reacted only against theta 5 GST, with a single band at M_r 27,500 kDa. Human MIF antisera reacted with human MIF and TRANSMIF strongly and with moderate intensity to the known theta-class GSTs, DCMD DM4, and rat liver GST 5-5 (Fig. 3). The theta GST 5-5 band visualized by the MIF antisera had the same mobility as that visualized by the theta 5 antisera. Purified rat liver alpha GSTs (1-1, 1-2, 2-2) and mu GSTs (3-3, 3-4, 4-4) did not cross-react with the anti-MIF antibodies.

Discussion

MIF was discovered independently by Bloom and Bennett (1966) and David (1966) as a lymphokine produced by

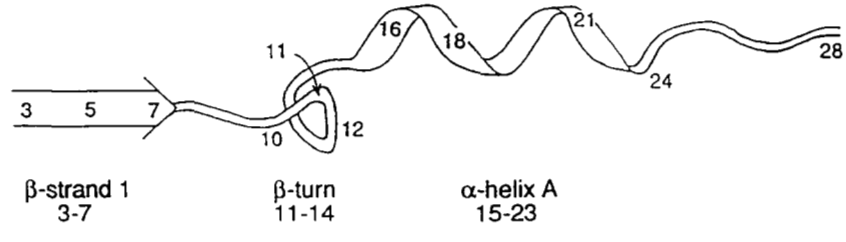

Fig. 2. The N-terminal motif that delineates GST superfamily gene classes with 100% sensitivity and specificity. The 11 pattern positions (Table 2) that uniquely define 5 GST classes overlaid upon a linearized ribbon diagram of the first three secondary structural elements from domain 1 of the pig pi GST structure (Reinemer et al., 1991).

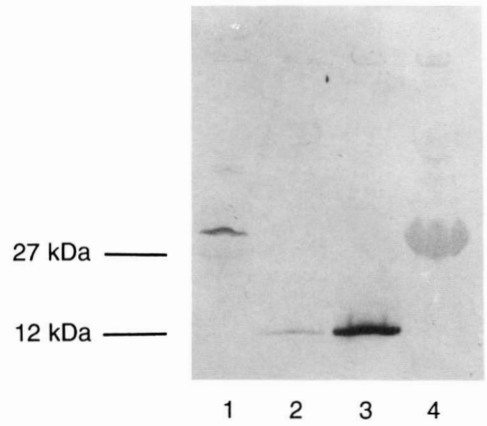

Fig. 3. Western immunoblots using goat anti-human MIF antisera. Gels were gradient 8–25% sodium dodecyl sulfate-polyacrylamide gels. Immunoblots were run as discussed in the Material and methods. Samples are as follows: theta GST 5-5 pool, lane 1; TRANSMIF, lane 2; human MIF, lane 3; and DM4 DCMD, lane 4.

antigen-stimulated T cells that retarded the random migration of mononuclear cells in the presence of specific antigen. MIF is now recognized as a principal cytokine modulating T-cell–macrophage interactions in the expression of delayed hypersensitivity and acquired cellular immunity (Rocklin et al., 1970). More recently, a recombinant human MIF has been shown to be responsible for the specific activation of macrophages to kill the intracellular parasites *Mycobacterium avium* and *Leishmania donovani*. Intracellular growth of *M. avium*, the principal bacterial agent infecting AIDS patients, is strongly inhibited by MIF (Orme et al., 1993). NO, a potent antimicrobial agent (Hibbs et al., 1990), is produced by the NO synthase-mediated oxidation of terminal L-arginine guanidine nitrogen atoms. Recombinant MIF activates macrophages to express NO synthase. The elevated levels of NO thus produced enhance the killing of intracellular *L. donovani* (Weiser et al., 1991; Cunha et al., 1993).

MIF proteins, however, have a broader role than these immediate aspects of cellularly acquired immunity. Proteins with high amino acid homologies to and of identical size with human MIF have been reported from a variety of disparate sources. Two are involved with the immune system. Tagaya et al. (1993) reported the action of a recombinant murine GIF, which is a negative regulator of IgE production. This protein is 98% homologous with human MIF over all 115 residues. Bernhagen et al. (1993) suggested that a 12-kDa pituitary MIF from mice plays a central role in the systemic response to inflammatory stimuli. This MIF is 96% homologous with human MIF over the 27 N-terminal amino acids. Others are involved with growth and differentiation. Wistow et al. (1993) demonstrated the expression of MIF in cells outside of the immune system; MIF proteins from the developing eye lens of embryonic chicken, newborn mouse, and fetal human have homologies with human MIF of 72, 90, and 99%, respectively, over all 115 residues. The delayed early response gene (Lanahan et al., 1992) from mouse 3T3 fibroblasts encodes the DER6 protein, which is 90% homologous with human MIF.

Insights into the structure of the MIF proteins can contribute significantly to understanding their function and evolution. Recently, a rat liver homolog of human MIF was shown to function as a GST with 1,2-epoxy-3-(*p*-nitrophenoxy)-propane or dichloromethane as substrates (Blocki et al., 1992). Subsequently, human MIF has been specifically bound and eluted from glutathione affinity matrices (David, 1993). These observations suggest structural linkage between the GST and MIF protein families.

The recent pi- and mu-class GST X-ray structures solved with bound substrate (Reinemer et al., 1991, 1992; Ji et al., 1992) indicate that 81% of the residues directly contacting glutathione in domain 1 are found in the first 26 residues. This allowed us to focus on this region to develop class-specific patterns to describe the N-terminal glutathione-binding motif. This approach differs markedly from previous efforts that compared similar proteins by direct amino acid alignments over the entire sequence. Our methods can be applied more easily to proteins of disparate size that might share a common N-terminal glutathione-binding motif.

The MIF proteins are approximately half the size of a single dimeric GST subunit but appear to contain an N-terminal GST glutathione-binding domain that most resembles those of the theta GST class. Previously, the MIF sequence had been matched directly with a rat liver mu GST, isozyme 3-3 (Blocki et al., 1992). In the current study, MIF proteins matched the theta pattern 100% and the mu pattern by less than 26%. The importance of the 11 positions chosen for these patterns is shown by the conservation of class-specific residues at those positions across the entire 54-member GST superfamily and by the role the N-terminal residues play in glutathione binding as shown by X-ray crystallography.

Pattern position 7 contains a potential hydrogen-bonding amino acid implicated in glutathione deprotonation for catalyzing nucleophilic addition reactions. In pi, mu, and alpha GSTs, tyrosine fulfills this function as demonstrated by X-ray crystallography or site-specific mutagenesis. In the present study (Table 2), tyrosine is found here in all alpha-, mu-, pi-, and four of six phi-class GSTs, but not in the theta-class members. Theta-class members contain an aliphatic hydroxyl group in place of the aromatic hydroxyl group. TRANSMIF shows GST activity of 7 μmol/min/mg of protein with 1,2-epoxy-3-(*p*-nitrophenoxy)-propane at neutral pH (Blocki et al., 1992). All theta GSTs and DCMD DM4 contain serine, but MIF proteins have threonine at this position. A tyrosine cannot activate glutathione in TRANSMIF or hu-

man MIF because neither have a tyrosine within their 26 N-terminal residues.

Computational evidence for the MIF–theta GST link was supported by experimental data. Anti-MIF antibodies cross-reacted weakly with a 27,500 M_r protein in a rat liver fraction enriched 25-fold for GST 5-5 (Fig. 3). That this fraction contained GST 5-5 was indicated by enzymatic assay and by reaction of an identical 27,500 band with anti-GST 5-5 antisera. The identity of the anti-MIF cross-reacting protein of 36,000 M_r is unknown. The MIF–theta linkage is strengthened by the relatively strong reaction of anti-MIF antibody with the bacterial theta GST known as group A dichloromethane dehalogenase (DCMD DM4). Thus, these data demonstrate a structural similarity between human MIF and theta-class GSTs. Purified rat liver alpha and mu GSTs did not react with anti-MIF antibodies. Further studies are in progress to determine the specific sequences of mammalian and bacterial GSTs reactive with the anti-MIF antibodies.

It has been suggested that the theta GST class is representative of an ancient progenitor GST gene, which may have originated from the endosymbioses of a purple bacterium, ultimately leading to the mitochondrion (Pemble & Taylor, 1992). The fact that the MIF proteins human MIF, TRANSMIF, DER6, pituitary MIF (Bernhagen et al., 1993), GIF (Tagaya et al., 1993), and embryonic eye lens MIFs from mouse and human (Wistow et al., 1993) are all matched by the theta-class GST-specific pattern identified in this study raises interesting questions regarding the origin of this family of proteins.

It seems likely that MIF proteins diverged early in evolution apart from the larger GSTs. In this context, it would be of interest to look for MIF-like proteins in non-mammals. A further interesting question, germane to MIF mechanism of action, is the role of glutathione binding in MIF protein biological function. It seems unlikely that the widespread MIF proteins would all maintain a vestigial theta-like glutathione-binding domain, so efforts need to be focused on the biological function of glutathione binding within the MIF protein family.

Materials and methods

Computational

The PIR32 and Swiss-Prot protein and EMBL DNA data bases were searched for sequences from the GST superfamily. Duplicate GST loci were omitted. Theta GST fragments S14345 and S14346 were included because they span the entire GSH-binding site (Reinemer et al., 1991). Seven GST sequences not present in the data banks were retrieved from the current literature (Table 1). Two sequences translated from EMBL cDNA loci, TAGSTA (from wheat, *Triticum aestivum*) and PPG (from flounder, *Pleuronectes platessa*), were excluded due to unproven GST functionality of these putative proteins of homologous sequence; S16178, a putative yeast (*Issatchenkia orientalis*) GST, was omitted for similar reasons.

The human MIF to which TRANSMIF shared primary sequence identity was 115 residues long. DER6 has 90% homology with human MIF: 102 of 115 residues are the same. Thus, TRANSMIF, human MIF, DER6, 50 cytosolic GST sequences (13 alpha, 7 pi, 18 mu, 6 theta, 6 plant), and DCMD DM4 (LaRoche & Leisinger, 1990) comprised the set of known GST superfamily members for this study. Loci names, sequence titles, and species of origin are shown in Table 1.

The primary computational resource used in this study was the Molecular Biology Computing Center (MBCC) in the College of Biological Sciences, University of Minnesota. Supporting software was provided by the Molecular Biology Computing Research Resource (MBCRR) of the Dana Farber Cancer Institute and Harvard University.

The PIR and Swiss-Prot protein data bases and the EMBL nucleic acid data base were available as part of the Intelligenetics Suite at the MBCC. These data bases were searched using the Intelligenetics modules FINDSEQ with the keyword "glutathione transferase" and QUEST with the five class-specific primary sequence patterns listed in Table 2.

Several tools provided by the MBCRR expedited sequence retrieval and editing. Clustering of sequences was based on the PIMA algorithm (Smith & Smith, 1990, 1992). Initial sequence alignments provided by PIMA and multi-alignment sequence editor (MASE) (Faulkner & Jurka, 1988) were manually refined based on X-ray crystal structures.

To assemble a collection of alternate residue groupings at given primary sequence positions that would be found in all GST superfamily members, 13 members were selected, one from each subcluster with a similarity score above 100. These loci span the diversity of the entire superfamily.

Immunochemistry

Polyclonal rabbit antisera to theta 5 GST were a gift from Meyer et al. (1991) and polyclonal goat antisera to human MIF (Weiser et al., 1989) were a gift from Dr. Monica Tsang of R&D Systems (Minneapolis, Minnesota). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was run with Gradient 8-25™ gels in conjunction with Pharmacia LKB's PhastSystem™. Immunoblots were effected with Pharmacia LKB's PhastTransfer Semi-dry Electrophoretic Transfer System according to the manufacturer's specifications as delineated in instruction manual catalogue 80-1300-01. Immunoblots were developed sequentially with primary antisera, biotinylated secondary antibodies (Pierce: catalogue 31820X [GxR-Biot.] and 31732X [RxG-Biot.]), avidin-conjugated alkaline phosphatase, and the nitro blue tetrazolium/5-bromo-4-chloro-3-

indoyl phosphate chromogenic pair according to Pharmacia development technique file 211 (catalogue 80-1307-72).

Protein purification

A 25-fold purified GST 5-5 sample was prepared according to Meyer et al. (1991). The ratio of 1,2-epoxy-3-(*p*-nitrophenoxy)-propane to dichloromethane dehalogenase activity was 16.8, consistent with that recorded by Meyer et al. (1991). DCMD DM4 was purified as specified by Scholtz et al. (1988).

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