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Structural Requirements for Glycolipid Antigen Recognition by CD1b-Restricted T Cells

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The human CD1b protein presents lipid antigens to T cells, but the molecular mechanism is unknown. Identification of mycobacterial glucose monomycolate (GMM) as a CD1b-presented glycolipid allowed determination of the structural requirements for its recognition by T cells. Presentation of GMM to CD1b-restricted T cells was not affected by substantial variations in its lipid tails, but was extremely sensitive to chemical alterations in its carbohydrate or other polar substituents. These findings support the view that the recently demonstrated hydrophobic CD1 groove binds the acyl chains of lipid antigens relatively nonspecifically, thereby positioning the hydrophilic components for highly specific interactions with T cell antigen receptors.

Human CD1 proteins are a family of nonpolymorphic transmembrane glycoproteins expressed in association with β_2-microglobulin on the surface of antigen-presenting cells (APCs) (*1*, *2*). Unlike antigen-presenting molecules encoded in the major histocompatibility complex that present peptide antigens to T cells, at least two human CD1 proteins (CD1b and CD1c) mediate specific T cell recognition of bacterial lipid and glycolipid antigens (*3*–*6*). Two classes of CD1-restricted lipid antigens—mycolic acids and phosphoglycolipids such as phosphatidylinositol mannosides (PIMs) or lipoarabinomannan (LAM) (*4*, *5*)—have been identified. To find other antigens presented by the CD1 system, we established additional T cell lines specific for mycobacterial lipid antigens. Analysis of the $CD4^-CD8^-$ $TCR\alpha\beta^+$ T cell line LDN5, isolated from a skin biopsy of a cutaneous reaction to *Mycobacterium leprae* antigen, revealed evidence for a third class of CD1-restricted lipid antigens (*7*).

LDN5 proliferated to only one lipid fraction separated by preparative thin-layer chromatography (TLC) from organic extracts of *M. leprae* and cross-reacted strongly with a lipid of identical retardation factor (R_f) extracted from *M. phlei* (*8*). TLC staining indicated that the lipid contained carbohydrate (anthrone positive) but not phosphate (molybdenum negative), distinguishing this antigen from the two previously described classes of CD1-restricted antigens. Proliferative responses to the purified glycolipid were observed only for LDN5, but not for a panel of 14 other T cell lines, ruling out a nonspecific T cell–stimulating activity (Fig. 1A). LDN5 lysed antigen-pulsed C1R B lymphoblastoid cells transfected with CD1b but not mock-transfected cells, indicating that the antigen-specific response was mediated by CD1b (Fig. 1B).

The structures of the lipid and carbohydrate moieties of the antigenic glycolipid were determined separately. The products resulting from alkaline hydrolysis of the antigen were partitioned and recovered separately from organic and aqueous phases. The organic phase lipids coeluted on high-pressure liquid chromatography (HPLC) with mycobacterial mycolic acids (*4*), and the aqueous phase contained a single product that was identified as glucose by gas chromatography (GC). This composition analysis suggested that the glycolipid antigen was glucose monomycolate (GMM), a previously described mycobacterial cell wall component consisting of a single glucopyranoside residue esterified at its sixth carbon to mycolic acid (*9*).

Electrospray ionization mass spectroscopy (ESI-MS) analysis of the intact glycolipid revealed a predominant ion at a mass-to-charge ratio (m/z) of 1382, corresponding to GMM containing a monounsaturated, C_{80} wax-ester mycolic acid (Fig. 1C) (*10*). GMM was separately isolated from trehalose dimycolate (cord factor) treated with aqueous acid, which released intact GMM by cleavage at the α-glycosidic linkage (*11*). Cord factor–derived GMM stimulated LDN5 with a dose response that was nearly identical to that of the GMM purified directly from *M. phlei* (Fig. 1D). Thus, the antigenic glycolipid recognized by LDN5 was isolated from three independent sources and shown to be GMM, the prototype for a third class of CD1-restricted antigens, mycolyl glycolipids.

We determined the role of the lipid portion of GMM in T cell recognition by isolating GMM from mycobacterial species that differ in mycolic acid composition. *Mycobacterium bovis* BCG, *M. fortuitum*, *M. smegmatis*, and *M. phlei* produce GMMs consisting of glucose esterified to mycolic acids that vary in acyl chain length and the presence or absence of R group substitutions, double bonds, and cyclopropane rings (*12*). LDN5 responded to each of these different GMMs at equivalent doses, indicating that the naturally occurring structural variations of the hydrophobic tails of the antigen were unlikely to determine specific T cell responses (Fig. 2A). This result was definitively confirmed by the CD1b-re-

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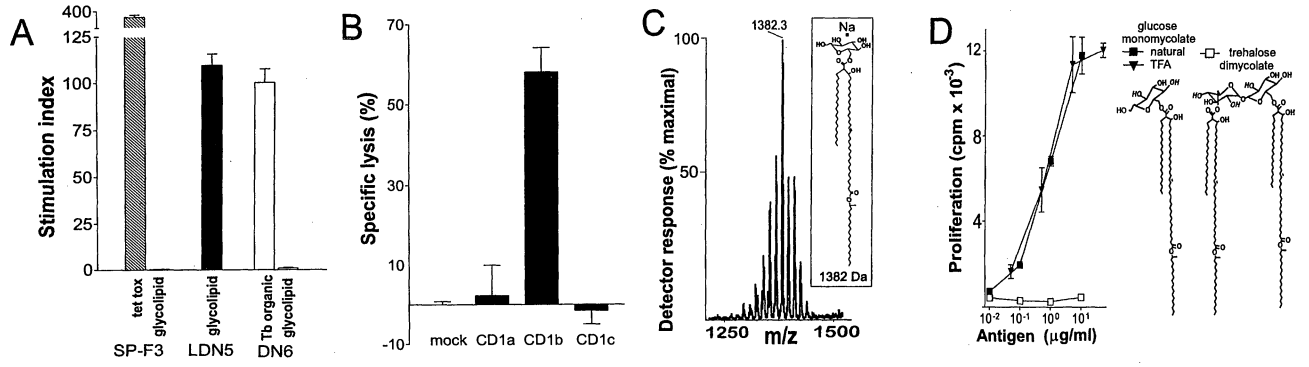

Fig. 1. Identification of glucose monomycolate as a CD1b-restricted T cell antigen. (**A**) The purified *M. phlei* antigenic glycolipid (2 μg/ml) presented by GM-CSF– and IL-4–activated monocytes stimulated LDN5, but not 14 other T cell lines tested, including SP-F3 [HLA-DR–restricted, tetanus toxoid–specific (tet tox); 10 μg/ml] and DN6 [CD1c-restricted, *M. tuberculosis* lipid–specific (Tb organic); 1/200 dilution], two examples shown here. Stimulation index was calculated as counts per minute in the presence of antigen per counts per minute in the absence of antigen (*6*). (**B**) LDN5 lysed CD1b-transfected C1R lymphoblastoid target cells (effector:target, 25:1) cultured with purified antigenic glycolipid (0.5 μg/ml), but not similarly treated mock, CD1a, or CD1c transfectants. No specific lysis was observed in the absence of antigen. (**C**) ESI-MS analysis of the intact antigenic glycolipid revealed two overlapping alkane series of ions corresponding to GMMs with mycolic acids containing either α or wax ester R groups and small variations in acyl chain length. The most abundant ion at m/z = 1382 was a Na adduct of GMM containing a monounsaturated C_{80} wax-ester mycolic acid. (**D**) LDN5 proliferated equally well in response to GMM independently isolated from two sources, "natural" GMM isolated directly from *M. phlei* and "TFA" GMM released on treatment of *M. phlei* trehalose dimycolate with TFA. LDN5 did not respond to intact trehalose dimycolate (cord factor). Similar results were obtained with GMM and trehalose dimycolate from *M. tuberculosis*.

stricted response of LDN5 to a fully synthetic GMM containing a C_{32} mycolic acid (Fig. 2, B and C) (*11*). This antigen lacked long chain length (compared with C_{80} mycolic acids of mycobacteria), cyclopropanation, double bonds, and R groups, ruling out all of these natural chemical variations of the mycolic acid moiety as necessary antigenic determinants.

The finding of a synthetic CD1-restricted antigen with a simple and well-defined structure allowed the systematic study of individual molecular features of the antigen that determined the specificity of the T cell response. Because the fine structure of the mycolic acid was not crucial for recognition by LDN5, we considered whether the spectrum of antigenic glycolipids might be extremely broad (any glucosylated lipid) or be limited to mycolyl glycolipids. Mycolyl glycolipids are defined by the α-branched, β-hydroxy structure of the mycolic acid, so analogs of GMM lacking these features were synthesized to test their role in T cell recognition (*13*). LDN5 did not respond to glucose 6-O-3-hydroxypalmitate, a GMM analog lacking the α-carbon branch (Fig. 2D). Likewise, removal or derivitization of the β-hydroxyl of the mycolic acid abolished the T cell response entirely (Fig. 2D). In addition, LDN5 did not respond to a variety of non–mycolyl glycolipids that were similar in structure to GMM, containing glucose linked to acyl chains of approximately 32 C atoms (*14*). Therefore, recognition of GMM was absolutely dependent on the α-branched, β-hydroxy lipid structure that defines mycolyl lipids, but the long distally substituted acyl chains found in many naturally occurring mycolic acids were not required.

The role of the carbohydrate moiety of the glycolipid in T cell recognition was separately evaluated. The CD1b-restricted response of LDN5 to GMM was carbohydrate dependent, because free mycolic acids were not antigenic (Fig. 3A). The carbohydrate moiety of GMM could in theory have contributed to antigenicity by facilitating APC uptake or processing of the antigen (*15*). Analysis with the CD1b-restricted T cell line DN1, which is specific for free mycolic acid (*4*), revealed that this was unlikely. DN1 responded to free mycolic acid and not to GMM, whereas LDN5 showed the opposite pattern of recognition of these two antigens (Fig. 3A). Thus, the

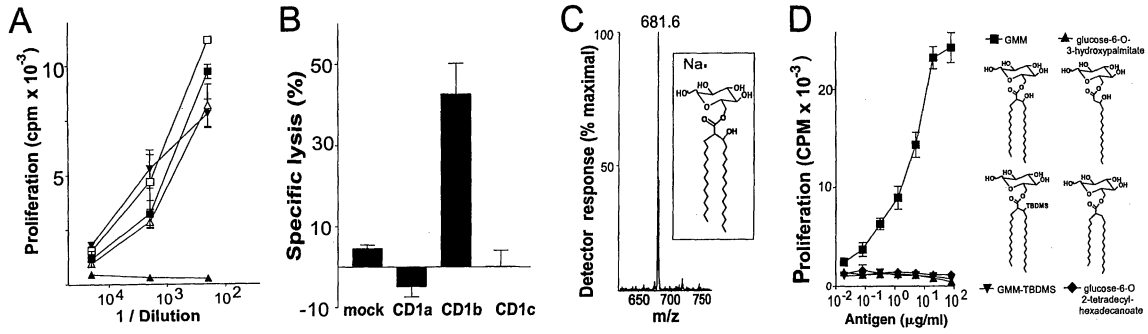

Fig. 2. The fine structure of the lipid moiety of GMM did not determine T cell recognition. (**A**) LDN5 proliferated in response to GMM from all strains tested [*M. phlei* (■), *BCG* (□), *M. smegmatis* (△), and *M. fortuitum* (▼)] but not to trehalose dimycolate (▲)]. These stimulatory GMMs contained mycolic acids that differed in R group composition and the presence of cyclopropane groups or double bonds (*12*). Antigen concentrations were normalized such that a dilution of 1:1 corresponded to the concentration of GMM recovered from preparative TLC of organic extract from 15 mg of each bacterium (*8*). (**B**) LDN5 lysed C1R lymphoblastoid target cells (effector:target, 25:1) transfected with CD1b and cocultured with synthetic GMM (5 μg/ml). Similarly treated mock, CD1a-, or CD1c-transfected cells were not lysed. (**C**) The Na adduct of a fully synthetic GMM containing C_{32} mycolic acid was detected by ESI-MS analysis as an ion peak at m/z = 681.6. This synthetic antigen lacks long chain length, R groups, unsaturation, and cyclopropanation. (**D**) LDN5 proliferated in response to synthetic GMM but not to analogs lacking either the α-branched (glucose-6-O-3-hydroxypalmitate) or β-hydroxy [3-*tert*-butyldimethylsilylated-GMM (GMM-TBDMS), glucose-6-O-2-tetradecylhexadecanoate] structure (*13*).

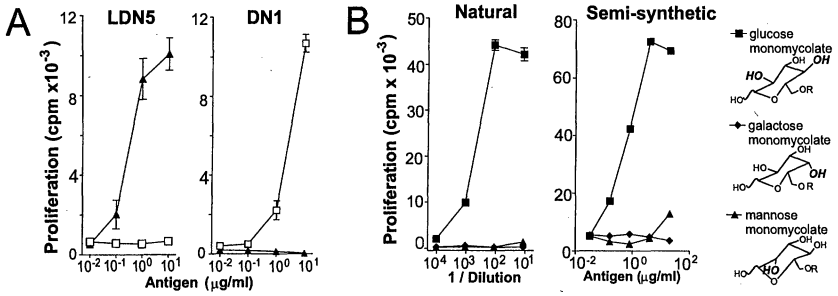

Fig. 3. Carbohydrate-specific recognition of mycolyl glycolipids. (**A**) The CD1b-restricted T cell lines DN1 and LDN5 demonstrated converse reactivities for *M. tuberculosis*–free mycolic acid (□) and GMM (▲) presented by CD1b-expressing activated monocytes. (**B**) LDN5 proliferated in response to natural or semisynthetic GMM at similar doses, but gave only a trace response to mannose monomycolate and no response to galactose monomycolate. These mannose- and galactose-containing mycolyl lipids differ from GMM only in the orientation of a single hydroxyl group at the 2 or 4 position on the pyranose ring (bold italics), respectively (*13*). R, *M. tuberculosis* α-mycolic acid.

carbohydrate dependence of GMM recognition by LDN5 was a specific feature of this CD1b-restricted T cell line, suggesting that the glucose component of the antigen was directly involved in T cell recognition of this glycolipid.

To investigate the specificity of the T cell response for the carbohydrate moiety of GMM, we purified a variety of differentially glycosylated mycolic acids. The structure of the carbohydrate was crucial for the T cell response, as LDN5 responded to M. *tuberculosis* GMM but not to M. *tuberculosis* mycolyl esters of glycerol, trehalose, and arabinose (*13*). To examine the T cell response to mycolyl glycolipids most similar to GMM, we prepared two stereoisomers of GMM, mannose monomycolate and galactose monomycolate (*13*). LDN5 proliferated at similar doses to natural and semisynthetic GMM. In contrast, LDN5 responded very weakly or not at all to mannose monomycolate and galactose monomycolate, epimers of GMM at the 2 or 4 positions of the pyranose ring (Fig. 3B). Thus, these T cells discriminated among stereoisomers varying only in the orientation of a hydroxyl group on the pyranose ring.

The identification of this third class of lipid antigens revealed a general motif for CD1b-restricted lipid antigens. Synthetic GMM is intermediate in structure between the two previously known antigens, PIM and mycolic acid. GMM contains a true mycolic acid, but like PIM is glycosylated. The long (C_{80}) and distally substituted lipid moiety of natural GMM was shortened (C_{32}) and simplified (Fig. 2C) to take on a form similar to that of the two saturated acyl chains of PIM without losing antigenicity, as long as the branched lipid structure was maintained (Fig. 2D). Thus, CD1b-restricted antigens from each of the three classes share a structural motif in which a single proximally branched acyl chain or two acyl chains are capped with a hydrophilic moiety (Fig. 4). The fine structure of the hydrophilic caps was crucial for T cell recognition, as changes in the the β-hydroxyl or pyranose ring of GMM (Figs. 2D and 3B), the free carboxylate of mycolic acid (Fig. 3A), or the carbohydrates of PIM and LAM (*5*) abolished the T cell response. The identification of this motif should guide the search for new foreign and potentially self lipid antigens. For example, these results demonstrate that glycolipids with short-chain mycolic acids characteristic of nonmycobacterial actinomycetes, including human pathogens such as *Corynebacterium diphtheriae* and *Nocardia asteroides*, can be presented by CD1b.

The recently solved crystal structure of a murine CD1d protein revealed that its α1 and α2 domains form a deep hydrophobic ligand-binding groove organized into two contiguous pockets, A′ and F′. The hydrophobic groove is connected to the exterior of the protein through a narrow opening lined by conserved charged and polar amino acids above the F′ pocket (*16*). Thus, in terms of size, shape, and electrostatic topography, the CD1 ligand-binding groove is ideally suited to interact with lipids conforming to the CD1b antigen motif with the two acyl chains buried within the A′ and F′ pockets. This mechanism of binding would leave the hydrophilic cap to interact with polar and charged amino acids at the entrance to the groove (Fig. 4) (*17*). In the case of CD1b presentation of GMM, this structural model places the β-hydroxyl and pyranose ring at the α-helical surface of CD1 that is predicted to interact with the T cell receptors (TCRs) of CD1b-restricted T cells. Thus, the specificity of LDN5 for the CD1b isoform and the precise structure of the carbohydrate of GMM can be accounted for by recognition of an epitope formed by the CD1 protein and the hydrophilic portions of GMM. This straightforward structural model provides a mechanism by which T cells specifically interact with unconstrained, hydrophobic lipid antigens in an aqueous environment. Detailed structural studies of this model will reveal how the immune system can specifically respond to a previously unrecognized universe of antigens that are normally buried within biological membranes.

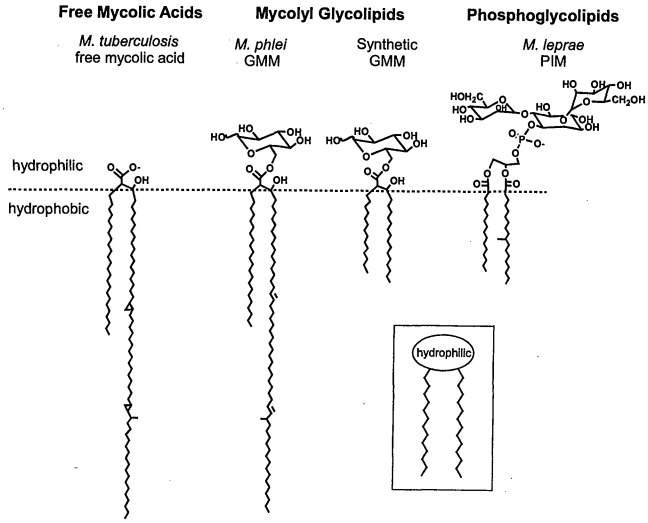

Fig. 4. Structural motif for CD1b-restricted antigens. Each of the known CD1b-restricted lipid antigens contains a proximally branched acyl chain or two acyl chains capped by a hydrophilic group (inset). T cells discriminate changes in the hydrophilic cap of members of each of these three classes of antigen (*4*, *5*) (Fig. 3A). For mycolyl glycolipids, T cells demonstrated fine specificity for the structure of the carbohydrate, β-hydroxy, and branched chain structure of the antigen (Figs. 2D and 3B), but not substantial differences in substitutions or length of the acyl chains (Fig. 2, A and C).

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7. LDN5 was derived from the same human skin lesion that gave rise to the previously described LAM-reactive T cell line LDN4 (*5*). Cultures were stimulated initially with autologous granulocyte-macrophage

colony-stimulating factor (GM-CSF)– and interleukin-4 (IL-4)–treated CD1$^+$ monocytes and *M. leprae* sonicate. After establishment of LDN5, cultures were maintained in IL-2–supplemented medium and periodically stimulated with allogenic CD1$^+$ APCs and *M. phlei* sonicate. Flow cytometric analysis of LDN5 revealed positivity for αβ TCR, but not CD4 or CD8β. T cell culture methods, proliferation assays, and cytolysis assays have been described (*6*).

8. *Mycobacterium phlei*, *M. tuberculosis H37Ra*, *M. fortuitum*, *M. smegmatis*, and *M. bovis BCG* were cultivated in 7H9 medium (Difco) supplemented with 0.05% Tween-80 and 1% glucose, mannose, or galactose. Organic extracts (1×) were made by shaking 7.5 mg of lyophilized bacteria per 1 ml of chloroform:methanol (2:1) at 20°C for 2 hours. Sonicates (1×) were made by probe sonication of 10 mg of bacteria per milliliter of phosphate-buffered saline, subsequently clarified by centrifugation as described (*6*). Mycolyl glycolipids were purified with preparative silica TLC in solvent A (60:16:2 chloroform:methanol:water) and extraction from silica into chloroform:methanol (2:1) or by eluting an open 2 cm by 20 cm silica column serially with chloroform and acetone in a stepwise gradient. The antigenic glycolipid eluted in 30% acetone in chloroform.
9. P. J. Brennan, D. P. Lehane, D. W. Thomas, *Eur. J. Biochem.* **13**, 117 (1969). The purified antigenic glycolipid was hydrolyzed, and the resulting products were partitioned between aqueous and organic phases. Organic soluble products were derivatized with phenacyl bromide and coeluted on C18 reversed-phase HPLC with *M. tuberculosis* mycolic acids as described (*4*). We determined the carbohydrate structure by methylating the reducing end of the intact glycolipid (0.5 N HCL in methanol at 65°C for 2 hours), followed by alkaline hydrolysis. Aqueous-phase products were acetylated and compared with acetylated methyl glycosides of authentic glucose and other carbohydrates by GC.
10. G. S. Besra and D. Chatterjee, in *Tuberculosis, Pathogenesis, Protection and Control*, B. R. Bloom, Ed. (American Society for Microbiology, Washington, DC, 1994), pp. 285–306. ESI-MS was performed on a Quattro II triple quadrupole mass spectrometer in the positive mode with samples in chloroform:methanol (2:1) at a flow rate of 2 to 4 μl/min.
11. We hydrolyzed *M. phlei*, *M. tuberculosis* (Sigma), and synthetic (Ribi) α,α′-trehalose dimycolate to yield GMM by drying on glass and treating with 2 M trifluoroacetic acid (TFA) at 121°C for 2 hours [G. S. Besra, T. Sievert, R. L. Lee, A. Slayden, P. J. Brennan, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 12737 (1994)]. The yield of the resulting glycolipids was determined by comparison of TLC with authentic GMM standards. ESI-MS analysis revealed ions of the expected *m/z* for GMM.
12. *Mycobacterium bovis BCG* and *M. tuberculosis* mycolic acids contain cyclopropyl groups, whereas *M. smegmatis* mycolic acids contain double bonds in place of cyclopropyl groups [K. Kaneda *et al.*, *J. Gen. Microbiol.* **134**, 2213 (1988); Y. Yuan, R. E. Lee, G. S. Besra, J. T. Belisle, C. E. Barry, *Proc. Nat. Acad. Sci. U.S.A.* **92**, 6630 (1995)]. These species produce mycolic acids containing either no R group (α and α′ mycolates) or named R groups as follows: *M. tuberculosis* (α, keto, methoxy); BCG (α, keto); *M. phlei* (α, wax-ester, and possibly small amounts of keto); and *M. fortuitum* and *M. smegmatis* (α, α′, epoxy) [(*18*); R. E. Lee, P. J. Brennan, G. S. Besra, *Curr. Top. Microbiol. Immunol.* **215**, 1 (1996)].
13. Previously described methods for trehalose monomycolate purification, mycolic acid synthesis, TBDMS derivatization, and hexose-6-*O*-acyl preparation [A. K. Datta, K. Takayama, M. A. Nashed, L. Anderson, *Carbohydr. Res.* **218**, 95 (1991)] were used, except that the appropriate lipid [3-hydroxypalmitate (Matreya), tetradecylhexadecanoate (Wako), or triacontanoate (Sigma)] or carbohydrate [glucose, mannose, or galactose (Sigma)] were substituted in the reactions. "Natural" hexose mycolates were isolated from *M. phlei* grown in glucose-, galactose-, or mannose-supplemented media [Y. Natsuhara, S. Oka, K. Kaneda, Y. Kato I. Yano, *Cancer Immunol. Immunother.* **31**, 99 (1990)]. Glycerol monomycolate (*18*), and arabinomycolate were purified from *M. tuberculosis* [G. S. Besra *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 12735 (1994)]. Lipid structures were confirmed by ESI-MS and TLC. Nuclear magnetic resonance analysis of semisynthetic hexose monomycolates (Bruker ACE-300) revealed a low-field chemical shift of H-6_a (δ 4.51, doublet) and H-6_b (δ 4.06, double doublet) indicative of acylation at the position 6 hydroxyl.
14. LDN5 failed to respond to glucopsychosine, *n*-lignoceroyldihydroglucocerebroside, *n*-palmitoyldihydroglucocerebroside, *n*-stearoyldihydroglucocerebroside (Sigma), and glucose-6-*O*-triacontanoate (*13*) at all doses tested.
15. LDN5 recognition of GMM appeared to require antigen uptake and processing, as was the case for CD1b-restricted recognition of mycolic acid (*3*, *4*) and lipoglycans (*5*). Treatment of macrophages with 25 mM chloroquine reduced the proliferative response of LDN5 to GMM by 52% compared with macrophages pulsed with antigen before chloroquine treatment. Treatment of macrophages with 0.025% glutaraldehyde before antigen exposure abolished the response of LDN5 to GMM (*3*).
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17. The CD1-lipid antigen association is predicted to occur with an orientation similar to that of phosphatidylcholine in the hydrophobic cavity (576 A^2 surface area) of bactericidal permeability-increasing protein (BPI) in which the acyl chains are buried deeply and the charged phosphate group is exposed to aqueous solvent [L. J. Beamer, S. F. Carroll, D. Eisenberg, *Science* **276**, 1861 (1997)]. The CD1 groove is larger (1310 A^2 surface area) and more extended than that of BPI, suggesting that the acyl chains of CD1 ligands may lie in an extended conformation rather that parallel as is the case for BPI (*16*). These interactions contrast with that of free fatty acids with fatty acid binding protein in which the free fatty acid is oriented with the charged carboxylate at the bottom of the groove stabilized by basic amino acid side chains [J. Eads, J. C. Sacchettini, A. Kromminga, J. I. Gordon, *J. Biol. Chem.* **268**, 26375 (1993)].
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Structure-Based Analysis of Catalysis and Substrate Definition in the HIT Protein Family

Christopher D. Lima, Michael G. Klein, Wayne A. Hendrickson

The histidine triad (HIT) protein family is among the most ubiquitous and highly conserved in nature, but a biological activity has not yet been identified for any member of the HIT family. Fragile histidine triad protein (FHIT) and protein kinase C interacting protein (PKCI) were used in a structure-based approach to elucidate characteristics of in vivo ligands and reactions. Crystallographic structures of apo, substrate analog, pentacovalent transition-state analog, and product states of both enzymes reveal a catalytic mechanism and define substrate characteristics required for catalysis, thus unifying the HIT family as nucleotidyl hydrolases, transferases, or both. The approach described here may be useful in identifying structure-function relations between protein families identified through genomics.

Human FHIT and PKCI proteins are members of the ubiquitous protein family HIT, which denotes a conserved histidine triad (His-x-His-x-His) sequence motif (*1*). HIT protein sequences have been identified independently and through genomics in Prokaryae, Archae, and Eukaryae [(*1*, *2*) and references therein]. Members can be aligned to reveal at least two subfamilies. One subfamily, of which human PKCI is a member, is found throughout evolution and is characterized by a highly conserved COOH-terminal sequence and greater than 94% amino acid sequence identity overall between known mammalian homologs. We previously described the identification, cloning, and structure determination of human PKCI in its unliganded form (*3*). Human FHIT, whose gene resides in a fragile locus on human chromosome 3 (*4*), is a member of a divergent eukaryotic HIT subfamily that differs significantly from the more conserved subfamily (~20% identity) by deviations at NH_2- and COOH-termini. Disruption of the *FHIT* gene is associated with human cancers (*4*), but definitive evidence supporting its role as a tumor suppressor has yet to be elucidated. We recently reported the three-dimensional structure of FHIT and its complex with a nucleoside ligand (*2*). A core domain of ~100 amino acids is similar among all HIT family members; PKCI and FHIT share 20.7% identity over a 110–amino acid overlap.

Characterized HIT proteins exist as ho-

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