CD1 proteins are antigen-presenting molecules that bind foreign and self-lipids and stimulate specific T cell responses. In the current study, we investigated ligand binding by CD1 proteins by developing a fluorescent probe binding approach using soluble recombinant human CD1 proteins. To increase stability and yield, soluble group 1 CD1 (CD1b and CD1c) and group 2 CD1 (CD1d) proteins were produced as single chain secreted CD1 proteins in which β2-microglobulin was fused to the N terminus of the CD1 heavy chains by a flexible peptide linker sequence. Analysis of ligand binding properties of single chain secreted CD1 proteins by using fluorescent lipid probes indicated significant differences in ligand preference and in pH dependence of binding by group 1 versus group 2 CD1 proteins. Whereas group 1 CD1 isoforms (CD1b and CD1c) show stronger binding of nitrobenzoxadiazole (NBD)-labeled dialkyl-based ligands (phosphatidylcholine, sphingomyelin, and ceramide), group 2 CD1 (CD1d) proteins were stronger binders of small hydrophobic probes such as 1-anilinonaphthalene-8-sulfonic acid and 4,4′-diaminobenzene-1,1′-naphthyl-5,5′-disulfonic acid. Competition studies indicated that binding of fluorescent lipid probes involved association of the probe with the hydrophobic ligand binding groove of CD1 proteins. Analysis of selected alanine substitution mutants of human CD1b known to inhibit antigen presentation showed that NBD-labeled lipid probe binding could be used to distinguish mutations that interfere with ligand binding from those that affect T cell receptor docking. Our findings provide further evidence for the functional specialization of different CD1 isoforms and demonstrate the value of the fluorescent lipid probe binding method for assisting structure-based studies of CD1 function.

CD1 is a family of β2-microglobulin-associated transmembrane glycoproteins that have substantial structural similarity to MHC I proteins. Unlike MHC I proteins that bind and present peptide antigens, CD1 proteins bind lipid antigens in their hydrophobic antigen binding grooves and initiate CD1-dependent, lipid-specific T cell responses (1). The five CD1 proteins expressed in humans are classified into three distinct groups based on amino acid homology and various characteristics of their expression and functions. Group 1 CD1 is composed of the CD1a, CD1b, and CD1c isoforms, and these are expressed primarily on myeloid lineage dendritic cells where they initiate adaptive immune responses against self or microbial lipid antigens (2–6). In contrast, the more structurally divergent CD1d protein is classified as group 2 CD1 and is expressed on most cells of hematopoietic origin and also on certain epithelia. CD1d is essential for the development of a unique subset of T cells known as natural killer T cells (NK T cells), which contribute significantly to innate immune responses and also appear to play an important role in immune regulation (7–9). Finally, the CD1e molecule may define a third separate group (i.e. group 3 CD1). It appears to be expressed mainly intracellularly or as a secreted protein, and its potential function is currently unknown (10).

At present, four classes of lipid antigens are known to be presented by CD1 molecules to T cells as follows: 1) mycolates (including free mycolic acids and glucose monomycolates from mycobacteria and related bacteria (6)); 2) diacylglycerols (mycobacterial lipoidalmannan, phosphatidylinositol, and eukaryotic glycosylphosphatidylinositol (5, 11)); 3) glycosphingolipids (gangliosides, sulfatides, and α-glycosylceramides (12–14)); and 4) glycosylphosphoinositol (mycobacterial mannosylated phosphoinositol (4)). All of these lipid antigens contain a common motif of a polar head group covalently linked to one or more hydrophobic alkyl chains. Studies using mutagenesis of CD1 and CD1-restricted TCRs in addition to recently solved crystal structures of CD1b and CD1a complexed

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1 The abbreviations used are: MHC, major histocompatibility complex; β2m, β2-microglobulin; scCD1, single chain CD1; 2,6-ANS, 2-anilino-6-naphthalene-8-sulfonic acid; 1,8-ANS, 1,8-anilinonaphthalene-8-sulfonic acid; bis-ANS, 4,4′-diaminobenzene-1,1′-naphthyl-5,5′-disulfonic acid, dipotassium salt; NBD-C12-HPC, 2-(12-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)dodecanoyl-sn-glycero-3-phosphocholine; NBD-C6-HPC, 2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl-sn-glycero-3-phosphocholine; NBD-C6-SM, 6-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino)hexanoyl-1-hexadecanoyl-sn-glycero-3-phosphocholine; NBD-C6-Cer, 2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino)hexanoyl-1-hexadecanoyl-sn-glycero-3-phosphocholine; NBD-C6-SM, 6-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl-1-hexadecanoyl-sn-glycero-3-phosphocholine; NBD-C6-Cer, 2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl-1-hexadecanoyl-sn-glycero-3-phosphocholine; NBD-C6-Cer, 2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-NBD-C12-HPC; NBD-C6-Cer, 2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl-phosphoinositol; αGalCer, α-galactosylceramide; βGalCer, β-galactosylceramide; LAM, lipoarabinomannan; GM3, glutamate non-mycolylate; HA, hemagglutinin; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; FBS, fetal bovine serum; IL, interleukin; NK, natural killer; mAb, monoclonal antibody; CHO, Chinese hamster ovary; TCR, T cell receptor; FACS, fluorescence-activated cell sorter; Ni-NTA, nickel-nitritolricetic acid.
Antigen Binding Properties of Human CD1 Proteins

with phosphatidylinositol or glycosphingolipids demonstrated that CD1 molecules present lipid antigens by anchoring their hydrophobicalkyl chains within the hydrophobic binding groove of the proteins (15, 16). This mechanism of binding positions the polar head group or hydrophilic cap of the bound lipid at or near the opening of the groove where it can make direct contacts with T cell antigen receptors (17–20). Because the alkyl tails of lipid antigens interact directly with the surface of the hydrophobic ligand-binding sites of CD1 proteins, it seems likely that structural differences in the size and shape of the binding grooves of different CD1 isoforms will influence the identity of the lipid ligands that they bind and present. For example, the crystal structure of CD1b revealed that its ligand binding region consists of a maze-like network of multiple inter-connected channels, which can accommodate very long hydrocarbon chains of CD1b antigens such as mycolates. In contrast, the binding grooves of the CD1a and CD1d proteins contain two hydrophobic pockets suited for binding the two relatively short alkyl chains present in all currently known lipid antigens presented by these two CD1 isoforms. The unique structure of mycobacterial lipid antigens presented by CD1c, which are mannosylated phosphosphoprenoids that contain a single alkyl tail with multiple methyl branches, suggests that the ligand binding region of CD1c may have slightly different properties than those of CD1b and CD1d.

Given the established role of lipid antigen presentation by CD1 molecules in the triggering and regulation of a wide variety of immune responses (21, 22), the development of better methods with which to probe the antigen binding properties of each of the different CD1 isoforms is required. In the current study, we have established a new experimental system using soluble recombinant CD1 proteins and lipophilic fluorescent probes to characterize the lipid binding properties of three different isoforms of human CD1 proteins. By using a range of structurally distinct fluorescent lipid probes, we show that different isoforms of CD1 differ significantly in terms of the ligand selectivity and the influence of pH on ligand binding. Our results show that fluorescent lipid probes can be used to characterize the selective ligand binding properties of different CD1 proteins, and support the view that the diversification of the CD1 family into multiple different isoforms represents significant and nonredundant differences in the functions of these isoforms.

MATERIALS AND METHODS

Cell Lines and Antigens—Chinese hamster ovary (CHO), 293, 293T, and HeLa cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). CD1-transfected HeLa cells and 293 cells have been described previously (17, 23). JRT.3.T.15, a T cell receptor (TCR) β chain-deficient mutant of the Jurkat T leukemia line, was also obtained from ATCC. Mouse NK T hybridoma DN32.D3 was a generous gift of Dr. A. Bendelac and has been described previously (24). CHO and HeLa cells were maintained in Dulbecco modified Eagle’s medium (catalog number 10313021, Invitrogen) containing 10% FBS. All T cell lines were maintained in RPMI medium 1640 (catalog number 11835040, Invitrogen) supplemented with 10% FBS and the additional additives as described previously (23). The total lipid extracts of Mycobacterium tuberculosis and purified Mycobacterium phlei glucose 6-phosphomonoesterase (GMM) were prepared according to methods published previously (20, 25). Purified liposanabinominan (LAM) of M. tuberculosis was provided by Drs. John Bolis and Patrick Brennan (Colorado State University, Fort Collins, CO). The CD1d-presented NK T cell ligand α-galactosylceramide was produced synthetically, and has the structure [2S,3S,4R]-1-0-(α-D-galactopyranosyl)-N-tetrasacceroyl-2-amino-1,3,4-octadecanetriol. Sulfate and β-galactosylceramide purified from bovine brain were purchased from Sigma.

Antibodies and Fluorescent Probes—Monoclonal antibodies (mAbs) were used as either mouse ascites fluid or purified IgG isolated from hybridoma culture supernatants using protein G affinity chromatography (Pierce and Amersham Biosciences). CD1b-specific mAbs used in this study were BCD1b (mlgG1 (17)), BCD1b3 (mlgG1 (26)), BCD1b5 (mlgG1 (17)), BCD1b6 (mlgG1 (17)), AT3-6.5 (mlgG2a (27)), Nu-T2 (mlgG1 (17)), and WM-25 (mlgG1 (28)). CD1c-specific mAbs used were F10/2A3 (mlgG1), F10/21A3 (mlgG1 (33)), IGH3 (mlgG1 (29)), L616 (mlgG1, Beckman-Coulter), NCL-CD1C (mlgG2a, Novacstra, Newcastle upon Tyne, UK), M241 (mlgG1, ID Lab, London, Canada), and PHM-3 (mlgG2a, RDI, Flanders, NJ). CD1d mAbs were all described previously (30) and included CD1d27 (mlgG1), CD1d42 (mlgG1), CD1d51 (mlgG2b), CD1d75 (mlgG1), CD1d68 (mlgG1), and CD1d69 (mlgG1). Anti-human β2-microglobulin (β2m)-specific mAb, MAC1115 (mlgG1), was purchased from Sercote (Oxford, UK). All of the fluorescent probe molecules used in this study were purchased from Molecular Probes (Eugene, OR). The following probes were used: 2-anilinonaphthalene-6-sulfonic acid (2,6-ANS); 1-anilinonaphthale-6-sulfonic acid (1,8-ANS); 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonylic acid, dipotassium salt (bis-ANS); 2-(12-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)dodecanoyl-1-hexadecanoyl-sn-glycero-3-phospholipine (NB-D-C12-HPC); 2-(6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexadecanoyl-1-hexadecanoyl-sn-glycero-3-phospholipine (NB-D-C6-HPC); 6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoylphilipsoylphospholipine (NB-D-C6-SM); and 6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoylphosphosine (NB-D-C6-Cer). Fluorescent probes were dissolved at 1 mM concentration in ethanol or methanol according to the manufacturer’s instructions.

Generation of Single Chain CD1 Proteins—Single chain CD1 (scCD1)
cDNA constructs were generated by linking the sequence for the entire human β2m at its C terminus to the N-terminal sequence of the α1 domain of the CD1 proteins by the insertion of a sequence encoding a flexible peptide linker (GGGGSOSOGSGA). Sequence elements encoding influenza hemagglutinin (HA) epitope tag (GLVPRGRSPY-DVPDYAIEG), a BirA enzymatic biotinylation site (HVGLNDFEIAQK-IEWHEGH), and a hexahistidine (HHHHHH) tag were added to the C terminus of the α3 domain of the CD1 proteins as is diagrammed in Fig. 1.

Briefly, scCD1 expression constructs were generated in several steps by assembling fragments of DNA encoding the different functional elements. The pcDNA3.1 eukaryotic expression plasmid (Invitrogen) was adapted with complementary double-stranded synthetic oligonucleotides designed with an NarI restriction enzyme site (GGGCGCC) preceding the DNA encoding the influenza HA epitope tag, the BirA enzymatic biotinylation site, and a hexahistidine tag ending in a stop codon. A separate pcDNA1.1 vector was created with an insert that encodes the entire human β2m cDNA flanked by the glycine- and serine-rich linker ending in an NheI restriction enzyme site (GTCAGC). PCR was performed using human Jurkat T cell poly(A) RNA template to obtain a DNA fragment preceded by a KpnI restriction enzyme site (GGCGC) that encodes the human β2m leader and mature protein and containing without the stop codon into the Gly/ Ser linker ending in an NheI restriction enzyme site (GTCAGC). The oligonucleotides used for this PCR were 5′-GGTACCACTACTGTCCTGCCTGGTGCCTCG-3′ and 5′-CCTAGTGCCATCCCTCCGAGGATCCACTGTCCTGCC-3′. Separate PCRs were performed using plasmids containing the full-length human CD1b, CD1c, or CD1d isoform cDNAs as templates to extract cDNA fragments that encode forms of each of the mature CD1 proteins that would start from the N terminus of the α1 region without signal peptide and terminate just prior to the transmembrane segment. The forward direction oligonucleotides for these PCRs were designed to have an in-frame NheI restriction enzyme site (GTCAGC) that encodes the human β2m leader and mature protein and containing without the stop codon in to the Gly/Ser linker ending in an NheI restriction enzyme site (GTCAGC). The specific oligonucleotides pairs used for each of these reactions are as follows: CD1b, 5′-GGAG-CTTACGAGCAATGCTTCCCTCAAGGGGCGACCTCC-3′ and 5′-AGCCGCAATTTGGAGGAGTGGTGTTCCCTGCGATAGG-3′; CD1c, 5′-GGAGCTGCACTGGGCACACGATCCATGGCCTCCTCC-3′ and 5′-AGATGATCACATTGTAGCCTGCTGCTGCTGCTGCTGCTGCT-3′; and CD1d, 5′-GGAGCTTACGCTGATAAGCCACAGGCTCCAGAGGCTCCCTCC-3′ and 5′-AGGCCCGCCCAAGCCCATGAGGTCAGTACCCTCCACGC-3′. A three-part ligation reaction was performed to join the β2m and linker portion of the construct with the CD1 cDNA and the epitope tag elements in the pcDNA3 expression vector. The resulting scCD1 constructs were fully sequenced to ensure that the coding regions and junctions contained no mutations.

CHO cells were transfected with scCD1/pcDNA3 vector constructs

*S. Porcelli, unpublished data.
containing a neomycin/G418 resistance marker using LipofectAMINE (Invitrogen). G418-resistant cells were selected and subcloned by limiting dilution, and culture supernatants were screened by capture ELISA to isolate stably transfected cells, which produced proteins at a high level. Stable CHO transfectants were adapted to CHO-S-SFM II media (Invitrogen, catalog number 12052098) and cultivated using a roller bottle system to scale up the production. One liter of culture supernatants was extensively dialyzed against 4 liters of PBS for 2 days with buffer exchange every 8 h, and then imidazole was added to the final concentration of 20 mM. The culture supernatants were passed by gravity through Ni-NTA affinity column (Qiagen, Valencia, CA), which contained 2 ml of Ni-NTA beads. CD1 proteins were eluted using 1 ml of PBS containing 200 mM imidazole, and the elution fractions were subjected to gel filtration using G200 size exclusion chromatography (Pharmacia). The purified soluble mouse CD1d protein with noncovalently associated β2m was produced using a previously published baculovirus expression system that was kindly provided by Dr. Mitchell Kronenberg (31). Protein concentration was determined by following formula: \( \Delta A_{280} \times M_{r} \times \text{molar extinction coefficients} \). Molar extinction coefficients for scCD1b, -c, and -d, based on the content of tyrosine and tryptophan residues, were calculated to be 96,690, 97,970, and 116,770 liter/cm/mole, respectively. The purity of proteins was assessed by reducing SDS-PAGE analysis. The deglycosylation of proteins was performed using peptide-N-glycosidase F (New England Biolabs, Beverly, MA) according to the manufacturer’s instructions.

Single chain CD1 mutant constructs were generated by replacing the wild type CD1b sequence between the BamHI and NotI sites of the scCD1b/pCDNA3 construct with analogous fragments containing the desired alanine substitution mutations. The mutant CD1b fragments were amplified by PCR from the previously reported CD1b mutant constructs (17, 32) by using the following primers: 5’-ggatccggttctggaggtggaggttcagaacatgcctcccag and 5’-gcggccgcctcacaggatgtcctg for CD1c; and 5’-gcggccgcctcacaggatgtcctg for CD1d. The resulting scCD1b mutant constructs were fully sequenced to confirm the specific mutations and were used to produce stable CHO transfectants as described above. Soluble mutant scCD1b proteins were purified from culture supernatants using Ni-NTA affinity chromatography and size exclusion fast protein liquid chromatography. The CD1 proteins were eluted using 1 ml of buffer exchange every 8 h, and then imidazole was added to the final concentration of 20 mM. The CD1 proteins were eluted using 1 ml of Ni-NTA beads. CD1 proteins were eluted using 1 ml of buffer exchange every 8 h, and then imidazole was added to the final concentration of 20 mM.

Animals and Tissue Culture—For T cell stimulation assays, scCD1 mutant constructs were used to generate the plate-bound CD1d-lipid complexes. scCD1d proteins at a concentration of 0.5 μM (25 μg/ml) in 6 μl were pre-complexed in solution with various molar excesses of αGalCer or βGalCer for 1 h at 37°C and diluted into 150 μl using PBS to a protein concentration of 0.02 μM (1 μg/ml). Then 50 μl of diluted CD1d-lipid complexes was used to coat one well of 96-well plates in triplicate at a protein concentration of 0.02 μM (1 μg/ml) in 100 μl complete medium containing 2% FBS. After washing with PBS to remove unbound lipids and proteins, DN32.D3 mouse NK T hybridoma cells (5 × 10⁴/well) were added in 200 μl of complete medium and incubated for 24 h. Supernatants were then harvested and assayed for the level of murine IL-2 using standard capture ELISA (PharMingen, San Jose, CA).

For assays to study the ability of various lipids to competitively block the ability of αGalCer by scCD1d (Fig. 6B), βGalCer was used as a control. To study the ability of various lipids to competitively block the ability of αGalCer by scCD1d (Fig. 6B), βGalCer was used as a control. To study the ability of various lipids to competitively block the ability of αGalCer by scCD1d (Fig. 6B), βGalCer was used as a control. To study the ability of various lipids to competitively block the ability of αGalCer by scCD1d (Fig. 6B), βGalCer was used as a control. To study the ability of various lipids to competitively block the ability of αGalCer by scCD1d (Fig. 6B), βGalCer was used as a control.

Circular Dichroic Spectral Analysis—Circular dichroic spectral analysis was performed on a Jasco J-815 spectropolarimeter (Jasco, Great Dunmow, UK) using a quartz cuvette with a path length of 1 cm. The spectra were recorded from 5 μg of CD1 proteins in pH 4 citrate buffer at various pH values at room temperature as 1 scan at 50 nm/min with a 1-nm step. The data were reported as the mean residue ellipticity [θ] in units of degree cm²/dmol from 190 to 240 nm at a 1-nm interval. The content of α-helices was calculated using a secondary structure estimation analysis program (Jasco).
**RESULTS**

**Construction, Expression, and Purification of Single Chain CD1 Proteins**—The association with β2m in the endoplasmic reticulum is required for assembly of CD1b proteins and for the subsequent transport to the cell surface (35–37). This observation led us to modify the structure of CD1 proteins by covalently linking β2m to the N termini of CD1 via a flexible (G₄S)₃ linker to create fully assembled secreted CD1 proteins with a single chain CD1 structure (scCD1, Fig. 1A). This single chain structure would be expected to accelerate the secretion of the soluble protein by increasing the rate of assembly in the endoplasmic reticulum and also to stabilize soluble CD1 proteins by reducing the rate of β2m dissociation. Stable transfectants expressing scCD1 proteins were cultivated by using low protein, serum-free medium that enhanced the quality of purification by Ni-NTA-agarose chromatography. Previous reports (38) have indicated that recombinant proteins expressed by CHO cells may generally contain fewer or simpler glycan structures than those expressed in human cells because of a relative lack of sugar-transferring enzymes. This potential for lower glycosylation of scCD1 proteins using the CHO expression system may be advantageous for in vitro ligand binding studies because large glycans may interact in vitro directly with the hydrophilic portion of CD1 ligands.

Following the initial recovery of soluble scCD1 proteins from culture supernatants by Ni-NTA affinity chromatography, size exclusion chromatography showed that the majority of proteins were expressed as monomers (Fig. 1B). Purified single chain CD1 proteins migrated in reducing SDS-PAGE as moderately diffuse bands that generally had molecular weights slightly greater than those calculated for their unmodified peptide sequences (calculated molecular masses: CD1b, 49.55 kDa; CD1c, 49.970 kDa; and CD1d, 50.197 kDa), whereas native mouse CD1d proteins produced in the baculovirus system with noncovalently bound β2m migrated as two bands consistent with the predicted molecular weights (34.511 kDa for heavy chain and 11.687 kDa for β2m). The broad bands of scCD1 proteins on SDS-PAGE were consistent with the addition of 2 to 3 N-linked glycans, as deglycosylation gave rise to tightly packed bands on SDS-PAGE (Fig. 1C). Essentially all of the material in the scCD1 protein preparations could be enzymatically biotinylated as determined by the streptavidin binding assay (data not shown), thus indicating the absence of contaminating proteins. The final yields of soluble scCD1 proteins in this expression system ranged from 5 to 20 mg/liter culture supernatant.

**Demonstration of Correct Folding of Single Chain CD1 Proteins**—To determine whether the purified scCD1 proteins maintained the correct folding and conformation of their natural two-chain counterparts, their binding to a panel of conformation-sensitive anti-CD1 mAbs was assessed by direct ELISA using anti-β2m antibody MCA1115 as a positive control (Fig. 2). All anti-CD1b mAbs bound specifically to recombinant scCD1b proteins at a level comparable with anti-β2m mAb binding, suggesting that scCD1b proteins were correctly folded (Fig. 2). The binding of anti-CD1c mAbs, F10/2A3 and 10C3, and anti-CD1d mAbs, CD1d51 and CD1d89, to scCD1b was...
consistent with the known cross-reactivity of these mAbs with cell-surface expressed CD1b (see Ref. 39 and additional data not shown). All the anti-CD1c and anti-CD1d mAbs also recognized recombinant scCD1c and scCD1d proteins, respectively (Fig. 2). Interestingly, scCD1d protein was recognized by mAb CD1d75, which was shown previously to bind denatured human CD1d proteins but could not bind to native cell-surface expressed CD1d (30). This suggests that the epitope recognized by CD1d75 is located on the extracellular portion of the proteins but is masked in cell surface expressed CD1d proteins. In summary, the strong and specific binding of multiple anti-CD1 mAbs to the solid phase bound scCD1 proteins implied that these were correctly folded and conformationally intact.

Biophysical Analysis of scCD1 Proteins Using Spectropolarimetry—Circular dichroism spectra were obtained to investigate the three-dimensional structures of scCD1 proteins (Fig. 3). This showed that all of the scCD1 proteins contained the typical mixed \( \alpha \)-helical and \( \beta \)-sheet structure seen in MHC class I-like molecules, consistent with a correctly folded structure. Interestingly, there was a subtle but clear distinction between group 1 and group 2 CD1 in terms of estimated \( \alpha \)-helical contents. At pH 7, the group 1 CD1 proteins (CD1b and CD1c) contained a similar level of \( \alpha \)-helical structure (24.9 and 25.1%, respectively), whereas the group 2 CD1d protein contained only 21.0%. This implies that group 2 CD1 diverges significantly from group 1 CD1 not only in the level of amino acid sequence homology but also in tertiary structure. However, all of the scCD1 proteins showed a similar degree of decrease in \( \alpha \)-helical contents upon acidification. This suggested that partial unfolding of the \( \alpha \)-helices making up the entry to the CD1 ligand binding groove may be a common feature of all CD1 isoforms that promotes the efficient loading of the lipid ligands in acidic environments such as endosomes. The estimated \( \alpha \)-helical structure of scCD1b was higher than previously reported for the CD1b crystal structure, which was 15.7% (15). The discrepancy may arise from the differences in methodology (i.e. measurement of \( \alpha \)-helical contents in solution state as opposed to crystal state) or because scCD1b contained a linker peptide, as well as HA and His\(_6\) tags, that may have augmented the \( \alpha \)-helical content.

Intact Antigen Presenting Functions of Soluble scCD1 Proteins—Although the scCD1 proteins appeared to be conformationally intact based on mAb binding and biophysical analysis, it remained necessary to show that they could bind relevant lipid ligands and be recognized by the TCRs of specific CD1-restricted T cells. To demonstrate this, membrane-bound forms of scCD1 containing the transmembrane and cytoplasmic domains were expressed on the surface of 293T or HeLa cells (Fig. 4A). These transfectants were then assessed for their ability to present lipid antigens to specific T cells. By using lipid antigen-specific Jurkat transfectant lines restricted by CD1b (LDN5/JRT3, specific for GMM) or CD1c (CD8–1/JRT3, specific for \( M. \) tuberculosis mannose phosphoisoprenoid) and a murine NK T cell hybridoma restricted by CD1d (DN32.D3, specific for \( \beta \)-GalCer), we found that each of the scCD1 molecules could efficiently present lipid antigens. Presentation by each of the cell surface expressed scCD1 molecules showed dependence on the antigen dose, and the levels of responses generated with scCD1 molecules were comparable or greater than that seen with native (i.e. two chain) cell surface expressed CD1 (Fig. 4, B–D). The differences in potency for antigen presentation observed between the scCD1 proteins and their native counterparts were to some extent accounted for by different levels of surface expression (Fig. 4A). Nevertheless, these results
showed that scCD1 molecules are capable of binding and presenting specific lipid antigens to CD1-restricted T cells in a grossly normal fashion.

Soluble scCD1d molecules were also evaluated for their antigen-presenting function using the αGalCer-reactive murine NK T cell hybridoma, DN32.D3. Soluble scCD1d proteins coated on tissue culture plate wells were able to present αGalCer but not βGalCer to mouse NK T cells in a dose-dependent manner (Fig. 4E). This provides further evidence that the scCD1d proteins were structurally and functionally intact.

**Binding of Nonpolar Hydrophobic Fluorescent Probes to scCD1 Proteins**—ANS and its analogs become fluorescent when bound to hydrophobic regions of proteins. This environment-sensitive property makes them valuable tools for studying protein folding or conformational changes (38, 40). A previous binding study using 1,8-ANS supported the conclusion that the antigen binding groove of recombinant scCD1b is hydrophobic and exposed upon acidification through the partial unfolding of α-helices (41). Here we extended this observation to other CD1 isoforms using ANS analogs of various sizes and conformations, and we used these fluorescent probes to compare the properties of the antigen binding regions of group 1 and group 2 CD1 isoforms.

As predicted by previous studies using native two-chain CD1b proteins, we found that 1,8-ANS bound to soluble CD1b proteins and that this was strongly augmented by acidic pH (Fig. 5B). In fact, all three of the scCD1b proteins tested showed enhanced binding of 1,8-ANS at acidic pH, although the increase in binding relative to the signal obtained at neutral pH was greater for group 1 (scCD1b and scCD1c) than for group 2 (scCD1d) proteins (Fig. 5C). The binding of 1,8-ANS to scCD1b proteins was strongly inhibited by preincubation of scCD1b proteins with the CD1b-presented glycolipid antigens, LAM and GM1, respectively (Fig. 5D, and additional data not shown). The blocking of 1,8-ANS binding to scCD1b by physiological CD1b ligands was consistent with the majority of the probe signal originating from the binding of 1,8-ANS in the antigen-binding site of scCD1b.

We also studied the binding of the more extended and larger probes 2,6-ANS and bis-ANS to each of the scCD1 proteins. In general, 2,6-ANS, which is more extended and less compact than 1,8-ANS, bound poorly to all scCD1 proteins, although a low level of binding could be detected which was increased at pH 4. In contrast, the larger bis-ANS showed increased levels of binding compared with 1,8-ANS at neutral pH, and even greater augmentation of binding at pH 4 (Fig. 5E). As for 1,8-ANS, the enhancement of binding of bis-ANS at acidic pH was greater for group 1 than for group 2 scCD1 proteins. Taken together, these results provided further support for the use of ANS derivatives as probes to analyze the binding properties of CD1 proteins. In addition, the results confirmed the pH effect on the binding of hydrophobic ligands to the CD1 groove and suggested that group 2 CD1 proteins may be less sensitive to this effect than group 1 CD1.

**Binding of NBD-conjugated Bi-alkyl Lipid Probes to scCD1 Proteins**—Many of the natural ligands that are bound and presented by CD1 proteins are lipids and glycolipids that contain two alkyl chains. Functional and structural studies demonstrate that these ligands are bound with their hydrophobic alkyl tails buried in the hydrophobic antigen binding groove of the proteins, and that this positions the polar head groups of such ligands so that they protrude from the opening of the groove where they can interact directly with the TCRs of CD1-restricted T cells (15, 20). In order to study lipid binding by scCD1 proteins by using probes that more accurately replicated the structure of natural CD1-presented antigens, we used a series of lipid probes containing two alkyl tails with a fluorescent NBD group at one end of one alkyl tail should allow the probe to become detectable if the antigen is correctly oriented in the CD1 groove.

To verify that fluorescent lipid probes could occupy the antigen binding grooves of the CD1 proteins, we tested the ability of each of the NBD-containing probes to block the binding of αGalCer to scCD1d (Fig. 6B). In order to do this, the lipid probes were pre-complexed with CD1d proteins, and the preformed complexes were then coated onto the surface of the wells of 96-well tissue culture plates. After washing to remove unbound lipids and proteins, αGalCer was added and incubated for 1 h at 37 °C to allow binding to unoccupied lipid binding grooves or displacement of bound NBD-coupled ligands. The murine αGalCer-reactive mouse NK T cell hybridoma, DN32.D3, was used to measure the extent of αGalCer association with the plate-bound scCD1d. This experiment revealed

**Fig. 3. Biophysical analysis of CD1 proteins using CD.** Circular dichroic spectra were recorded in the far UV range as detailed under “Materials and Methods.” The pH values of the samples were adjusted by adding 1 N HCl, and spectra were measure immediately. The α-helical content of each isoform was calculated using a secondary structure estimation analysis program (Jasco). A, CD spectra of scCD1b at different pH values. B, α-helical percentage of scCD1 proteins at different pH values. C, changes in α-helical contents of scCD1 proteins as percentage of value at pH 7.
FIG. 4. Functional analysis of scCD1. The antigen-presenting functions of transfected cells expressing either native CD1 proteins (i.e., with noncovalently associated β2m) or scCD1 proteins were compared. A, FACS analysis of surface expression of membrane-bound scCD1 or native CD1 proteins. The y axis represents relative cell number, and the x axis is mean fluorescence intensity. B, responses of Jurkat transfectant LDN5/JRT3.T1.5 to the purified mycobacterial antigen GMM presented by 293T cells expressing native or scCD1b or untransfected 293T cells. C, responses of Jurkat transfectant CD8-1/JRT3.T1.5 to M. tuberculosis lipid extract containing the mannosyl phosphoisoprenoid antigen presented by HeLa cells expressing native or scCD1c or transfected with empty vector only (mock). D, responses of mouse NK T cell hybridoma DN32.D3 to the synthetic glycolipid antigen αGalCer presented by HeLa cells expressing native or scCD1d or transfected with empty vector alone (mock). E, stimulation of mouse NK T cell hybridoma DN32.D3 by plate-bound αGalCer-scCD1d or βGalCer-scCD1d complexes. T cell responses were assessed by IL-2 production using HT-2 bioassay for human IL-2 (A and B, responses shown as [3H]thymidine incorporation by HT-2 cells) and ELISA for murine IL-2 (D and E).
that two of the NBD-containing probes, NBD-C6-HPC and NBD-C6-SM, were able to inhibit the binding of αGalCer. These achieved a level of inhibition that approached that of sulfatide, a natural glycosphingolipid that has been demonstrated to be presented by CD1 proteins (13).

Studies of the fluorescence emission of the NBD-labeled probes when incubated with the various scCD1 proteins revealed that all of the probes were able to bind significantly to the proteins (Fig. 6C). However, there were marked differences in the extent of binding of the different probes. The highest fluorescence emission for scCD1b binding was achieved with NBD-C6-HPC, with lower levels for NBD-C12-HPC and NBD-C6-SM. The pattern of probe binding was similar for scCD1c, except that this group 1 CD1 isoform showed much stronger binding of NBD-C12-HPC and NBD-C6-HPC compared with CD1b. The binding of all probes to scCD1b and scCD1c was enhanced by acidic pH. In contrast, the group 2 scCD1d protein showed weaker probe binding in nearly all cases compared with the group 1 scCD1 proteins, and also much weaker enhancement of binding by acidic pH.

Binding of Fluorescent Lipid Probes to Mutant scCD1b Proteins—A substantial number of site-directed mutations in the α1 and α2 domains of human CD1b have been reported to affect specific lipid antigen presentation to CD1b-restricted T cells (17, 32). Thus far, it has not been determined directly whether the effects of these mutations are due to a reduction in binding of lipid antigens by CD1b or to interference with the stable docking of the TCR on the surface of CD1b. We took advantage of the fluorescent probe binding assay to investigate the mechanism by which two alanine substitution point mutations in CD1b (Y169A and D83A) cause a marked reduction in T cell responses to the lipid antigens GMM and mycolic acid. Both mutant forms of CD1b were produced as soluble single chain recombinant proteins using the same methods described for the production of wild type scCD1 proteins. The soluble mutant proteins eluted as single monomeric peaks on size exclusion chromatography (data now shown). In ELISA, the mutant proteins showed similar reactivity with seven different mAbs against conformational epitopes of CD1b, with the single exception of reduced binding of mAb BCD1b3 to the D83A mutant (Fig. 7B). The recent crystal structure of human CD1b demonstrated that the side chain of Tyr-169 contributes to the distal portion of the hydrophobic A’ pocket structure. In contrast, the side chain of residue Asp-83 is exposed to the surface and pointing upward from the α1 helix and is well positioned to serve as a TCR recognition contact point (Fig. 7A). The binding of all NBD-conjugated fluorescent probes was significantly reduced for the Y169A scCD1b mutants compared with wild type scCD1b protein, whereas the binding to D83A scCD1b mutant proteins was not affected (Fig. 7C). These results provide the first direct evidence that mutations of individual amino acids
that contribute to the structure of the CD1b groove surface can alter the lipid ligand binding properties of the groove. This method thus provides a direct approach to determine whether alterations of T cell recognition by mutated or otherwise modified CD1 proteins are associated with changes in their binding properties.

**DISCUSSION**

CD1 proteins are known to bind lipids and glycolipids in a manner that leads to the formation of complexes that can be recognized directly by the clonotypic antigen receptors of particular T lymphocytes. However, the various parameters affecting lipid binding by CD1 remain largely unexplored, particularly for the human group 1 CD1 proteins that appear to play a significant role in the immune response to mycobacterial pathogens. Progress in this area has been slowed by the lack of simple and reliable methods for directly measuring the association of lipid ligands with CD1 proteins. Several previous studies (31, 41, 42) have examined lipid binding properties of group 2 CD1 molecules, using either measurement of surface plasmon resonance or responses of CD1d-restricted T cell hybridomas for detection of binding. In addition, one published study (41) has examined the binding of a small number of glycolipid antigens by recombinant human CD1b proteins, also using the Biacore device for measurement of surface plasmon resonance. These studies represent important initial steps in characterizing the lipid binding properties of CD1 proteins, but they were limited in scope and relied on methods that were inherently difficult to perform. As a step toward overcoming this limitation, the current study has evaluated the utility of various readily available fluorescent lipid probes in the study of ligand binding by recombinant soluble CD1 proteins.

Because of the difficulties inherent in refolding CD1 proteins produced in bacterial expression systems, we chose to express secreted forms of CD1 in mammalian CHO cells. By engineering these proteins to contain a His6 tag at their C termini, the proteins could be easily purified without exposure to harsh denaturants. In addition, the fusion of the β2m subunit to the CD1 heavy chain through a flexible hydrophilic linker sequence most likely led to enhanced expression and stability of the product. Although we have not directly compared the scCD1 proteins to soluble versions that lack the covalent linkage of β2m, this strategy has been successfully used in previous studies for the production of soluble MHC class I and CD1 proteins (42, 43). We were able to achieve adequate levels of scCD1 proteins in the supernatants of transfected CHO cells to allow the isolation of sufficient quantities of these proteins for ligand binding studies without requiring a large scale-up of culture volumes. The purified scCD1 proteins were soluble monomeric proteins that we found to be fully native in conformation and functional by a variety of criteria (Figs. 1–4).
Antigen Binding Properties of Human CD1 Proteins

Fig. 7. Characterization of CD1b mutant proteins using fluorescent lipid probe. Soluble D83A and Y169A scCD1b mutant proteins were generated to study their antigen binding properties using fluorescent lipid probes. Alanine substitutions at these residues were reported previously to show defective presentation of mycobacterial lipid antigens such as GMM and mycolic acid. A, crystal structure of human CD1b complexed with phosphatidylinositol as reported by Gadola et al. (15), with positions of side chains of Asp-83 and Tyr-169 indicated. Left panel shows view from above (i.e. looking down into the ligand binding groove), and right panel shows view from the side. Note that Asp-83 is located at the surface of the α1 helix and is thus positioned for interaction with residues of the TCR as it docks on the surface of the protein. In contrast, Y169A is positioned well below the surface of the protein and appears to contribute to the distal portion of the α2m pocket near the junction with the T tunnel. B, binding of anti-CD1b mAbs to mutant scCD1b proteins. Binding of mAbs was determined by ELISA as in Fig. 2. Bars show percent values for anti-CD1 antibodies normalized to the A405 obtained with anti-β2m MCA1115, (1(A405 of anti-CD1 − A405 of background)/A405 of MCA1115 − A405 of background)) × 100. All mAbs listed are specific for CD1b, except for M241, which is anti-CD1c and serves as a negative control for the binding assay. C, NBD probe-binding profile of D83A, Y169A, and wild type (WT) scCD1b proteins. Values are plotted as the percentage of the signal obtained for binding of each probe to the wild type scCD1b protein under the same conditions.

By using various analogues of ANS, a fluorescent probe that has been shown to be useful for measurement of binding to hydrophobic sites in proteins, we were able to demonstrate the presence of hydrophobic ligand-binding sites on all of the soluble scCD1 proteins. The signal generated by binding of ANS analogues showed a strong dependence on pH, with significantly enhanced fluorescence emission at acidic pH for all of the scCD1 proteins studied. This enhancement in ANS signal was appreciable over the range from pH 4 to 6, consistent with the normal pH of intracellular compartments within the endocytic system that have been implicated as the sites of antigen loading onto CD1 proteins in antigen-presenting cells (33, 35, 44). We also observed that the majority of fluorescence generated by addition of 1,8-ANS to scCD1b was inhibited by preincubation with equimolar amounts or moderate molar excesses of LAM and GM1, which are known CD1b-presented glycolipid antigens (Fig. 5D). This suggested that the ANS probe associated with the same ligand-binding site on the scCD1b proteins as the glycolipid antigens, which is most likely the large hydrophobic ligand binding groove in the α1 and α2 domains that has been identified by x-ray crystallography studies.

In general, the fluorescence emission signals generated by the addition of bis-ANS to scCD1 proteins were greater than those produced by 1,8-ANS and 2,6-ANS, possibly indicating that the size and conformation of bis-ANS provided a better fit for antigen binding grooves of the proteins (Fig. 5E). In fact, only bis-ANS, but not 1,8-ANS or 2,6-ANS, was able to block the association of αGalCer to scCD1d proteins, supporting this view (data not shown). Accordingly, the strong interaction of bis-ANS with scCD1 proteins makes it a very sensitive probe to study the effects of a variety of parameters on the antigen-binding site. In experiments using bis-ANS, we observed the largest augmentation of binding by acidic pH for scCD1b. The higher level of bis-ANS fluorescence generated by interaction with scCD1b may be consistent with a larger volume of the ligand binding groove for this CD1 isoform, which is also indicated from the recent x-ray crystallography studies of human CD1b (15).

Previous studies of the pH dependence of ligand association with group 2 CD1d proteins have yielded conflicting results. Although a number of studies have provided support for the view that lipid binding to CD1d may occur preferentially in acidified endocytic compartments (23, 30, 42), in vitro studies using the Biacore method indicated that recombinant CD1d proteins could associate with αGalCer at neutral pH (31). By using 1,8-ANS, the current study confirmed the intrinsic ability of soluble scCD1d to bind hydrophobic ligands at neutral pH (Fig. 5, C and E). Thus, although all of the scCD1 proteins studies showed a strong augmentation of 1,8-ANS binding at acidic pH, only scCD1d showed an appreciable binding of this fluorescent probe at neutral pH. This ability of scCD1d to bind hydrophobic ligands with the moderate efficiency at neutral pH...
suggests that the ligand binding groove of this CD1 proteins may be more exposed to solvent under these conditions, as has been indicated by the recent comparison of group 1 and group 2 CD1 crystal structures (16). Taken together, these findings give further support for the view that group 1 and group 2 CD1 proteins have subtle differences in their requirements for optimal lipid loading, which may be relevant to the specialized roles that they play in the immune response (21).

The current study also established the feasibility of using NBD-derivatized lipid probes to study the binding of lipid ligands to scCD1 proteins (Fig. 6). These probes were either diacylglycerols or ceramide-type structures that, compared with the ANS probes, more accurately replicate the structures of actual antigens known to be bound to CD1 proteins and presented to T cells. Several of the NBD probes tested interacted particularly well with the scCD1b and scCD1c proteins in solution, and the detection of fluorescence from the NBD group indicated that the alkyl tail to which this group was attached was buried within a hydrophobic environment. This is consistent with the recent comparison of group 1 and group 2 CD1 proteins. Thus, the D83A mutation, which the CD1b crystal structure shows that Tyr-169 is located within the ligand binding groove, forming part of the hydrophilic head group and general backbone structure of ligands, can influence the association with CD1, making it unlikely that the Y169A mutation would interfere with lipid presentation by disrupting TCR contacts. More likely, this mutation alters the structure of the ligand binding groove so that binding or stable association with the lipid ligand is reduced, a conclusion that is directly supported by the reduced NBD probe binding data presented here. Thus, these initial results strongly support the validity of the fluorescent probe binding approach for analyzing relationships between protein structure and function. Further studies using these probes are now in progress on selected mutants of residues known to alter function of CD1b.

In contrast to ANS derivatives, NBD-conjugated di-alkyl lipid probes bound to group 1 scCD1 proteins stronger than to the group 2 scCD1 proteins. This was probably not due to the presence of a less hydrophobic binding pocket in CD1d, because the binding of non-specific hydrophobic ANS probes to scCD1d-generated signals equal to those for the group 1 scCD1 proteins. More likely, the binding pocket of CD1d was not large enough to easily accommodate NBD-conjugated alkyl chains, compared with those of CD1b or CD1c. According to this, we analyzed the binding of NBD-labeled probes showed a clear distinction in the probable mechanisms by which these two mutations interfere with presentation of lipid antigens by CD1b. Thus, the D83A mutation, which the CD1b crystal structure predicts to be located at the surface of the α helix and oriented upward toward the TCR-docking site, had no significant effect on fluorescent lipid probe binding. In contrast, the Y169A mutation, which is also known to interfere with presentation of both long chain (C80) and short chain (C32) forms of the mycobacterial glycolipid antigen GMM, was associated with marked reduction in the binding of all four NBD probes tested. The CD1b crystal structure shows that Tyr-169 is located within the ligand binding groove, forming part of the distal surface of the α' pocket. This location and orientation make it unlikely that the Y169A mutation would interfere with lipid antigen presentation by disrupting TCR contacts. More likely, this mutation alters the structure of the ligand binding groove so that binding or stable association with the lipid ligand is reduced, a conclusion that is directly supported by the reduced NBD probe binding data presented here. Thus, these initial results strongly support the validity of the fluorescent probe binding approach for analyzing relationships between protein structure and function. Further studies using these probes are now in progress on selected mutants of residues known to alter function of CD1b.

In summary, we have shown that fluorescently labeled lipid probes can be used to analyze the antigen binding properties of CD1 proteins. Our studies using this method demonstrated subtle but measurable variations in the lipid binding properties of the different CD1 isoforms, indicating a significant level of functional specialization for the different members of the CD1 family. In combination with the use of soluble scCD1 proteins described here, this method should significantly facilitate the analysis of lipid binding and presentation by CD1 proteins, thus providing a relatively simple in vitro system for probing the physiological function of this family of proteins and elucidating their potential role in the immune response.

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