Corrections

APPLIED PHYSICAL SCIENCES. For the article “Ionic contrast terahertz near-field imaging of axonal water fluxes,” by Jean-Baptiste Masson, Martin-Pierre Sauviat, Jean-Louis Martin, and Guillaume Gallot, which appeared in issue 13, March 28, 2006, of Proc Natl Acad Sci USA (103:4808–4812; first published March 17, 2006; 10.1073/pnas.0510945103), the authors note that in Fig. 1 the y scale is incorrect because of a unit conversion error. The corrected figure and its legend appear below. This error does not affect the conclusions of the article.

IMMUNOLOGY. For the article “Saposin B is the dominant saposin that facilitates lipid binding to human CD1d molecules,” by Weiming Yuan, Xiaoyang Qi, Pansy Tsang, Suk-Jo Kang, Petr A. Illarionov, Gurdyal S. Besra, Jenny Gumperz, and Peter Cresswell, which appeared in issue 13, March 27, 2007, of Proc Natl Acad Sci USA (104:5551–5556; first published March 19, 2007; 10.1073/pnas.0700617104), the authors note that the author name Petr A. Illarionov should have appeared as Petr A. Illarionov. The online version has been corrected. The corrected author line appears below.

Weiming Yuan, Xiaoyang Qi, Pansy Tsang, Suk-Jo Kang, Petr A. Illarionov, Gurdyal S. Besra, Jenny Gumperz, and Peter Cresswell

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NEUROSCIENCE. For the article “PSD-95 and PKC converge in regulating NMDA receptor trafficking and gating,” by Ying Lin, Teresa Jover-Mengual, Judy Wong, Michael V. L. Bennett, and R. Suzanne Zukin, which appeared in issue 52, December 26, 2006, of Proc Natl Acad Sci USA (103:19902–19907; first published December 18, 2006; 10.1073/pnas.0609924104), the authors note that in Fig. 1A and B, the same trace was inadvertently used for the after-TPA (Right) records. The corrected figure and its legend appear below. This error does not affect the conclusions of the article.

PHYSIOLOGY. For the article “Metabolic syndrome and obesity in an insect,” by Rudolf J. Schilder and James H. Marden, which appeared in issue 49, December 5, 2006, of Proc Natl Acad Sci USA (103:18805–18809; first published November 28, 2006; 10.1073/pnas.0603156103), the authors note that on page 18805, in line 2 of the Abstract, and again in the main text, left column, paragraph 3, line 2, “(Microsporidia, Apicomplexa)” should instead appear as “(Apicomplexa: Eugregarinorida).”
Saposin B is the dominant saposin that facilitates lipid binding to human CD1d molecules

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CD1d molecules bind lipid antigens in the endocytic pathway, and access to the pathway is important for the development of CD1d-restricted natural killer T (NKT) cells. Saposins, derived from a common precursor, prosaposin, are small, heat-stable lysosomal glycoproteins required for lysosomal degradation of sphingolipids. Expression of prosaposin is required for efficient lipid binding and recognition of human CD1d molecules by NKT cells. Despite high sequence homology among the four saposins, they have different specificities for lipid substrates and different mechanisms of action. To determine the saposins involved in promoting lipid binding to CD1d, we expressed prosaposin deletion mutants lacking individual saposins in prosaposin-negative, CD1d-positive cells. No individual saposin proved to be absolutely essential, but the absence of saposin B resulted in the lowest recognition of α-galactosylceramide by NKT cells. When recombinant exogenous saposins were added to the prosaposin-negative cells, saposin B was the most efficient in restoring CD1d recognition. Saposin B was also the most efficient in mediating α-galactosylceramide binding to recombinant plate-bound CD1d and facilitating NKT cell activation. Saposin B could also mediate lipid binding to soluble CD1d molecules in a T cell-independent assay. The optimal pH for saposin B-mediated lipid binding to CD1d, pH 6, is higher than that of lysosomes, suggesting that saposin B may facilitate lipid binding to CD1d molecules throughout the endocytic pathway.

antigen processing | lipid transfer proteins | natural killer T cells

CD1 molecules present lipid and glycolipid antigens to T cells (1). In humans, five CD1 isoforms have been identified and classified into two groups based on sequence similarity: CD1a, -b, and -c constitute group I, whereas CD1d forms group II. CD1e represents an intermediate between the two. Mice express only CD1d, but there are two homologous genes, CD1d1 and CD1d2. Diverse lipids, most derived from Mycobacteria, are presented by group 1 CD1 molecules. These include mycolic acid, glucose-monomycolate (GMM), phosphatidylinositol mannoside (2), lipoarabinomannan (LAM) and mannosyl-β-1-phosphoisoeprenoid, recently redesigned mannosyl-β-1-mycoketide (3). Both endogenous and exogenous lipids can be presented by human and mouse CD1d (4, 5).

Whereas group I CD1 molecules present lipids to T cells with diverse T cell receptors, CD1d-restricted T cells predominantly use an invariant rearranged α chain: Vα24 in humans and Vα14 in mice. Many CD1d-restricted T cells coexpress CD161, which is expressed mostly on natural killer cells, and are referred to as natural killer T (NKT) cells (6). NKT cells rapidly secrete an array of cytokines after activation, and, although they constitute <1% of total T cells, they exert a critical influence in a variety of situations, including cancer (7) and bacterial, viral, parasitic, and fungal infections (8–10) and autoimmune diseases (11).

Lipids must be extracted from membranes to bind to CD1d molecules, a process facilitated by lipid transfer proteins. CD1d is initially assembled in the endoplasmic reticulum (ER), and here, the protein involved is the microsomal triglyceride transfer protein (MTP). After binding self-lipids, such as phosphatidylcholine (PC) and phosphatidylinositol (PI), in the ER (12), CD1d molecules follow the secretory pathway to the cell surface and recycle between the plasma membrane and the endocytic system (13).

Access to the endocytic pathway is important for processing and presentation of lipid antigens by CD1d (14–16) and several lysosomal lipid transfer proteins, particularly the sphingolipid activator proteins (SAPs), facilitate lipid binding (14, 17, 18). SAPs are membrane-perturbing and lipid-binding proteins initially defined by their roles in sphingolipid degradation (19). Important SAPs are saposins A, B, C, and D, which are acidic, heat-stable, and protease-resistant glycoproteins of ≈8–11 kDa (19). They are proteolytically derived from a common precursor, prosaposin (PS), which is encoded by a single gene. Deficiency in saposin expression causes sphingolipid accumulation, leading to lipid-storage diseases.

The four saposins share a high degree of homology, but they have different lipid specificities. Saposin A is required for galactosylceramide degradation by galactosylceramidase-β-galactosidase. Mice lacking saposin A accumulate galactosylceramide and suffer from a form of Krabbe’s disease (20). Saposin B facilitates the degradation of a variety of lipids, including sulfatide by arylsulfatase A and globotriaosylceramide and digalactosylceramide by α-galactosidase A. Patients lacking saposin B accumulate these substrates in lysosomes (21). Saposin C is required for the degradation of glucosylceramide by glucosylceramidase-β-glucosidase, and patients lacking this saposin develop a juvenile form of Gaucher’s disease (22). Saposin D stimulates lysosomal ceramide degradation by acid ceramidase and, in vitro, sphingomyelin hydrolysis by acid sphingomyelinase. Saposin D-deficient mice accumulate ceramides in the brain and kidney (23).

The saposins apparently activate sphingolipid degradation by different mechanisms. Saposin C directly interacts with glucosylceramide-β-glucosidase and induces conformational changes in the enzyme (24). It also destabilizes membranes by supporting the interaction of the enzyme with membrane-associated substrate lipids (22, 25). Saposin B and GM2 activator protein act as

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The authors declare no conflict of interest.

Abbreviations: CD1, cluster of differentiation 1; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DHPE, dihexadecanoyl-sn-glycero-3-phosphoethanolamine; α-GalCer, α-galactosylceramide; NKT, natural killer T; PS, prosaposin; PSKO, prosaposin knockout.

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physiological detergents and show broad lipid specificity. They each form a shell-like homodimer with the two subunits having different conformations and enclosing a large hydrophobic cavity (26). A lipid extraction mechanism has been proposed in which the open conformation interacts with the membrane and induces a reorganization of the lipid alkyl chains. Extraction of the lipid substrate is accompanied by a change to the closed conformation, which exposes it to the degradative enzymes as a water-soluble saposin–lipid complex (19).

Saposin C, but not other saposins, is required for lipid presentation by human CD1b (17). Saposin C can extract LAM from membranes in vitro and appears to interact directly with CD1b molecules, presumably facilitating LAM transfer. Recombinant saposins can promote lipid exchange by mouse CD1d, and mice lacking the PS gene fail to develop invariant NKT cells (27). We previously showed that mouse PS-negative cells expressing human CD1d are impaired in their ability to stimulate human NKT cells (14). Here, we examine the roles of the individual saposin(s). We find that although all four saposins can promote lipid binding to CD1d to some extent, saposin B is the most efficient.

Results and Discussion

Generation of CD1d-Positive Cells Lacking Individual Saposins. Expression of human PS in mouse PS- fibroblasts [PS knockout (PSKO)1 and PSKO2] transduced with human CD1d enhanced their ability to present lipid antigens to NKT cells (14). To determine whether an individual saposin is responsible, we generated four cell lines expressing mutant PS constructs, each lacking one saposin. The retroviral constructs are shown schematically in Fig. 1A and are referred to as PSΔA, PSΔB, PSΔC, and PSΔD. Expression of the constructs in PSKO2.CD1d cells (14) was examined by Western blotting using antibodies against the individual saposins (Fig. 1B). Each cell line expressed three saposins and lacked the deleted one, whereas the cells transduced with wild-type PS expressed all four, and the cells transduced with empty vector (mock) expressed none. All the cell lines showed comparable CD1d surface expression levels (Fig. 1C). PS is proteolytically processed into mature saposins in a stepwise fashion (28), and we found that the PS deletion mutants were processed as efficiently as the wild type, shown in Fig. 1D for saposin C. Similar results were obtained for saposins A, B, and D (data not shown).

![Fig. 1. Generation of cell lines expressing PS deletion mutants. (A) Diagram of saposin deletions in the coding regions of PS mutants. In each mutant, one saposin is deleted. (B) Immunoblotting showing PS or mutant PS expression in PSKO2.CD1d cells. Cell lysates from fibroblasts expressing PS lacking individual saposins, wild-type PS or no PS (mock) were probed with rabbit antisera to saposins A, B, C, or D or with a rat anti-Grp94 antibody as a loading control. (C) Flow cytometry of PSKO2.CD1d cells transduced with wild-type or mutant PS constructs. Cells were stained with PE-conjugated mouse anti-human CD1d mAb 42.1: wild-type PS transduced cells (thick line), and PSΔA-, -ΔB-, -ΔC-, and -ΔD-transduced cells (solid, dotted, dashed and long dashed lines, respectively). Isotype control is in gray. (D) Immunoblot of PSKO2.CD1d cells expressing PS or the mutants showing processed mature saposin C. The cell lysates used in B were probed with rabbit antiserum to saposin C.](www.pnas.org/cgi/doi/10.1073/pnas.0700617104 Yuan et al.)
unprocessed forms observed by Western blotting in Fig. 1 staining with the reactive saposin-specific antibodies, which probes the processed forms. In all the cells, we detected weak diffuse intense staining in the endosomes/lysosomes probably represents no prosaposin (mock) were used as antigen-presenting cells and incubated remaining saposins still colocalized with Lamp-1. The majority of pressing the mutants, the deleted saposin was absent, whereas the

To determine whether the elimination of individual saposins affected the subcellular localization of the remaining ones, we examined the cells by confocal immunofluorescence microscopy. In cells expressing wild-type PS, all four saposins colocalized with the late endosomal/lysosomal marker, Lamp1 (Fig. 2A). In cells expressing the mutants, the deleted saposin was absent, whereas the remaining saposins still colocalized with Lamp1. The majority of the saposins are completely or partially processed (Fig. 1D), and the intense staining in the endosomes/lysosomes probably represents the processed forms. In all the cells, we detected weak diffuse staining with the reactive saposin-specific antibodies, which probably corresponds to the ER (Fig. 2). This is likely to reflect the unprocessed forms observed by Western blotting in Fig. 1B and D.

Saposin B Most Efficiently Enhances CD1d Lipid Presentation to NKT Cells. We examined the ability of the cell lines expressing the deletion constructs to present the lipid α-GalCer to an NKT cell clone. Although all mutant PS species were able to enhance recognition by NKT cells, the cell line lacking saposin B was the least stimulatory (Fig. 3A), suggesting that saposin B is the most efficient enhancer of CD1d lipid loading. However, the combination of saposins A, C, and D expressed by PSΔ8B supported NKT cell stimulation to some extent. Recognition by NKT cells in the absence of α-GalCer was similar in all cases, consistent with the previous suggestion that saposins are not necessary for recognition of endogenous lipids (14). To determine whether any of the saposins could function alone, we reconstituted saposin expression in PSKO1.CD1d cells by adding purified recombinant saposins (Fig. 3B), previously shown to be functional SAPs in vitro (29). Confocal

Fig. 3. Saposin B enhances lipid loading to CD1d most efficiently. (A) PSKO2.CD1d cells expressing different PS mutants (PSΔA, ΔB, ΔC, and ΔD) or no prosaposin (mock) were used as antigen-presenting cells and incubated with either vehicle control (v.c.) or α-GalCer (GalC, 100 ng/ml). After fixation, 6F5 NKT cells were added, and IFN-γ secretion was measured by ELISA after 24 h. (B) Purified recombinant saposins (A–D) were separated by SDS/PAGE (15%) and detected by staining with Coomassie Brilliant blue. (C) Recombinant saposins are endocytosed and delivered to late endosomal/lysosomal compartments. PSKO1.CD1d.mock cells incubated with recombinant saposin C were stained with rabbit anti-saposin C and rat anti-mouse Lamp1 antibodies, followed by Alexa Fluor 488-conjugated goat anti-rabbit and Alexa Fluor 594-conjugated goat anti-rat Ig antibodies. (D) PSKO1.CD1d.mock cells were preincubated with saposins (A–D or A+B+C+D) overnight before incubation with α-GalCer. Control cells were left untreated and incubated with vehicle control (v.c. or α-GalCer (GalC)). After fixation, the cells were cocultured with 6FS NKT cells for 24 h, and IFN-γ secretion was determined by ELISA.

Saposins Directly Activate Lipids for CD1d Presentation. Saposin C appears to interact directly with CD1b to facilitate lipid binding (17). However, we were unable to detect a direct interaction between saposins and CD1d (data not shown). To investigate whether saposins directly mediate lipid binding to CD1d, we used a plate-bound CD1d-based lipid presentation assay (30). Human CD1d, expressed as an Fc-fusion protein in CHO cells and purified as described (30), was coated onto the wells of a 96-well plate, and α-GalCer was added in the presence or absence of recombinant saposins at pH 7.0. NKT cells were then added, and the response was determined by measuring the release of IFN-γ. Although there was some response in the absence of saposins, the addition of any saposin enhanced presentation of the lipid to the NKT cells (Fig. 4D). However, saposin B was clearly the most efficient, consistent with the results obtained with the PS deletion mutants (Fig. 4A).

Immunoassay microscopy showed that the added saposins accumulated in late endosome/lysosomal compartments, shown in Fig. 5C for saposin C. Addition of any of the four saposins enhanced CD1d presentation of α-GalCer to NKT cells (Fig. 3D). However, saposin B was the most efficient, consistent with the results obtained with the PS deletion mutants (Fig. 4A).

Fig. 4. Saposins directly activate lipids for CD1d presentation. (A) Recombinant human CD1d-Fc fusion protein was coated in triplicate in 96-well microtiter plates and incubated with vehicle control (v.c.), α-GalCer (GalC, 100 ng/ml) or α-GalCer in the presence of saposins A, B, C, or D at pH 7.0. After 48 h, 6FS NKT cells were added, and IFN-γ secretion was assayed by ELISA after a further 24 h. (B) Titration of molar ratio of saposin B: α-GalCer in the T cell stimulation assay using plate-bound CD1d. CD1d-Fc protein coated onto the wells of a 96-well plate was incubated with indicated amount of saposin B and α-GalCer (100 ng/ml), and IFN-γ secretion by the NKT cell line was measured. (C) CD1d-Fc-coated plates were incubated with α-GalCer in the absence (No saposins) or presence of one saposin (A–D) at indicated pHs for 48 h. T cell culture and analysis of secreted IFN-γ in the supernatant were as described in A and B.

Saposin B supported NKT cell recognition by NKT cells, the cell line lacking saposin B was the most efficient enhancer of CD1d lipid loading. However, the combination of saposins A, C, and D expressed by PSΔ8B supported NKT cell stimulation to some extent. Recognition by NKT cells in the absence of α-GalCer was similar in all cases, consistent with the previous suggestion that saposins are not necessary for recognition of endogenous lipids (14). To determine whether any of the saposins could function alone, we reconstituted saposin expression in PSKO1.CD1d cells by adding purified recombinant saposins (Fig. 3B), previously shown to be functional SAPs in vitro (29). Confocal immunofluorescence microscopy showed that the added saposins accumulated in late endosome/lysosomal compartments, shown in Fig. 5C for saposin C. Addition of any of the four saposins enhanced CD1d presentation of α-GalCer to NKT cells (Fig. 3D). However, saposin B was clearly the most efficient, consistent with the results obtained with the PS deletion mutants (Fig. 4A).

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lipid loading throughout the endocytic pathway, rather than exclusively in lysosomes. Substantial amounts of PS are secreted before endocytosis (37), and lipids, CD1d, and saposin B could meet at many points after endocytosis depending on where mature saposins are generated.

Exposure of the hydrophobic domains of saposins A, C, and D, and hence their affinity for phospholipid membranes, depends on low pH, but the hydrophobicity of saposin B apparently does not (38). The affinity of saposin B for lipid bilayers is low at both neutral and acidic pH, consistent with its function as an extractor/solubilizer that has only transient interactions with the membrane. Furthermore, low pH affects the structure of saposin B differently from the way it affects saposin A or C (35). Saposins A and C, as well as most other saposin-like proteins, have a compact, monomeric fold that buries a small hydrophobic core, whereas saposin B, like GM2 activator protein, has a major conformational variant that forms a homodimer that has a large hydrophobic cavity (26). For saposins A and C, low pH and the presence of detergent is required for dimer formation, whereas saposin B is dimeric at both neutral and low pH and in the presence or absence of detergent (35). These data again support the argument that the two categories of saposins interact differently with lipids in a manner relevant to the superior capacity of saposin C to facilitate lipid binding to CD1d.

Saposins are required for development of mouse invariant Vα14 NKT cells, indicating that they are involved in presenting self-lipids from thymocytes to developing NKT cells (27). The sphingolipid isogloboside b3 (Gb3) has been reported to be a self-antigen, and saposin B mediated the binding of Gb3 to mouse CD1d in a cell-free system (18). Another self-antigen identified by both human CD1d and CD1b is PI (33). There have been many reports that CD1d can be loaded with phospholipids and present them to T cells (30, 33, 39, 40). Saposin B, despite the fact it was initially identified as a sphingolipid activator protein, also binds and transfers phospholipids (41). It binds to some phospholipids with comparable or even higher affinities than it binds glycosphingolipids (34). Recombinant saposin B copurified with PE (26), and saposin B can mediate DHPE binding to CD1d (Fig. 5). Although the size and diversity of the self-lipid repertoire involved in NKT development and immune stimulation remains unclear, saposin B is likely to be involved in their presentation to NKT cells by CD1d during development.

Materials and Methods

Cell Lines and Antibodies. PSKO1 and PSKO2 cells stably expressing CD1d (PSKO1.CD1d, PSKO2.CD1d) and derivatives expressing human prosaposin were described (14). Four new cell lines were produced from PSKO2.CD1d by transduction with retroviruses encoding prosaposin with the regions encoding saposins A, B, C, or D deleted. For each construct, two internal primers were used to delete the appropriate coding region by PCR. The mutant genes were cloned into the pLPCX vector, and retrovirus was generated and cell lines selected as described (42). The human CD4+Va24+NKT cell line, 6F5, was a gift from Steven Porcelli (Albert Einstein College of Medicine, Bronx, NY) (43). Rabbit antiserum to saposins A, B, C, and D were described (32). Phycocerythrin-conjugated mouse anti-human mAb 42.1 and rat anti-mouse Lamp1 mAb were from BD–Pharmining (San Jose, CA). Alexa Fluor 594-conjugated goat anti-rat Ig antibody was from Molecular Probes (Eugene, OR).

Reconstitution of Prosaposin-Deficient Fibroblasts. Recombinant human saposins, expressed in Escherichia coli as His-tagged proteins, were purified as described (29). PSKO1.CD1d cells were preincubated with the saposins at 20 μg/ml for 18 h before addition of α-GalCer (100 ng/ml) or vehicle control, and NKt cell stimulation assays were performed as described (14, 42). IFN-γ secretion was assayed after 24 h by sandwich ELISA (BD–Pharmining). All experimental samples were in the linear range of the standard curve, and results are presented as mean ± standard deviation of triplicate samples.

T Cell Stimulation Assay Using Plate-Bound CD1d. Recombinant human CD1d-Fc (10 μg/ml) (44), was coated on Immulon plates overnight at 4°C. After washing, α-GalCer (100 ng/ml) or vehicle control was added with or without saposins for 48 h at 37°C, followed by washes with PBS and T cell medium. One hundred thousand 6F5 T cells were added to each well, and IFN-γ secretion was measured by ELISA after 24 h.

Lipid-Binding Assays. Biotinylated DHPE (Molecular Probes) or biotinylated α-GalCer, (25, 3S, 4R)-1-O-(α-d-galactopyranosyl)-2-[N-2-[2-(2-biotinylamino-ethoxy)-ethoxy]-ethoxyethyl]-tetracosanolymono-1,3,4-octadecatrienol, was sonicated in TBS (pH 5.5) in the presence or absence of detergent or saposins at the indicated concentrations, and soluble CD1d, purified as previously described (33), was added. After 1 h at 37°C, the samples were neutralized, diluted in TBS (pH 7.4), and neutravidin-agarose beads added (Pierce, Rockford, IL). After 1 h at 4°C and washing, the bound proteins were separated by SDS/PAGE, and CD1d binding was analyzed by immunoblotting with the anti-CD1d antibody, D5. To examine the effect of saposins on preloaded lipids, CD1d was incubated with biotinylated lipid in the presence of 0.5% CHAPS and isolated by using neutravidin-agarose beads as described above. The beads were then incubated with indicated reagents at 37°C for 1 h before washing and analysis by immunoblotting.

Flow Cytometry, SDS/PAGE, and Immunoblotting. These procedures were performed as described (42).

Confocal Immunofluorescence. Confocal immunofluorescence was performed as described (15, 42). Briefly, cells were seeded on sterile coverslips and allowed to adhere overnight. They were then fixed and permeabilized with 3% paraformaldehyde for 15 min at 4°C and then washed with PBS. The coverslips were incubated with the saposins at 20 μg/ml for 20 min at 4°C, washed with PBS, and then incubated with biotinylated lipid in the presence of 0.5% CHAPS. To examine the effect of saposins on preloaded lipids, CD1d was incubated with biotinylated lipid in the presence of 0.5% CHAPS and isolated by using neutravidin-agarose beads as described above. The beads were then incubated with indicated reagents at 37°C for 1 h before washing and analysis by immunoblotting.

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