A specific ligand for β_2-glycoprotein I mediates autoantibody-dependent uptake of oxidized low density lipoprotein by macrophages

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Abstract **β_2-Glycoprotein I (β_2-GPI) is a major antigen for antiphospholipid antibodies (Abs) present in patients with the antiphospholipid syndrome (APS). We previously reported that β_2-GPI specifically binds to oxidized low density lipoprotein (oxLDL), but not to native low density lipoprotein (LDL). In the present study, a ligand specific for β_2-GPI, oxLig-1, was purified from the extracted lipids of oxLDL. The structure of oxLig-1 was shown to be identical to that of synthesized 7-ketocholesteryl-9-carboxynonanoate by mass spectroscopy and nuclear magnetic resonance analyses. Both purified and synthesized oxLig-1 were recognized by β_2-GPI and subsequently by anti-β_2-GPI auto-Abs, either in enzyme-linked immunosorbent assay (ELISA) or in ligand blot analysis. Binding of liposomes containing oxLig-1 (oxLig-1-liposomes) to mouse macrophages, J774A.1 cells, was relatively low, as compared with that of phosphatidylserine (PS)-liposomes. In contrast, binding of oxLig-1-liposomes was enhanced more than 10-fold in the presence of both β_2-GPI and an anti-β_2-GPI auto-Ab (WB-CAL-1), derived from (NZW x BXSB) F1 mouse, an animal APS model. Anti-β_2-GPI auto-Abs derived from APS patients with episodes of arterial thrombosis were detected in ELISA, using a solid phase oxLig-1 complexed with β_2-GPI. We suggest that autoimmune atherogenesis linked to β_2-GPI interaction with oxLDL and Abs may be present in APS.**—Kobayashi K., E. Matsuura, Q. Liu, J. Furukawa, K. Kaihara, J. Inagaki, T. Atsumi, N. Sakairi, T. Yasuda, D. R. Voelker, and T. Koike. **A specific ligand for β_2-glycoprotein I mediates autoantibody-dependent uptake of oxidized low density lipoprotein by macrophages.** ***J. Lipid Res.*** **2001.** 42: **697–709.**

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The appearance of antiphospholipid antibodies (Abs) (aPL) such as anticardiolipin Abs (aCL) and lupus anticoagulants (LA) was found to be associated with thromboembolic manifestations such as cerebral or myocardial infarctions, venous and/or arterial thrombosis, recurrent fetal loss, and occasional thrombocytopenia in patients with systemic lupus erythematosus (SLE) (1–3). A subgroup of patients with these features was classified as having antiphospholipid syndrome (APS). aPL were initially considered to be directed to phospholipids (PLs), but now it is widely considered that some PL-binding proteins are true antigens for aPL. In 1990, three groups of investigators (4–6) independently reported that aCL were directed to β_2-glycoprotein I (β_2-GPI) complexed with cardiolipin (CL). Evidence has accumulated that β_2-GPI is a major antigen for aCL induced in patients with APS (7, 8). We also reported that aCL may recognize an epitope exposed on β_2-GPI that is conformationally altered by binding to an oxygenated polystyrene plate (9).

β_2-GPI, a glycoprotein with a molecular mass of 50 kDa and present in plasma at approximately 200 μg/ml, is composed of 326 amino acids and consists of five homologous domains, i.e., short consensus repeats (10). Four of them are composed of approximately 60 amino acids and each domain has two disulfide bridges. The fifth domain (domain V) contains 82 amino acid residues and three disulfide bridges. β_2-GPI binds to negatively charged substances such as PLs (11), heparin (12), and plasma membranes of activated platelets and apoptotic cells on which phosphatidylserine (PS) is exposed (13, 14). The PL binding core sequence, K^{282}NKEKK^{287}, was

Abbreviations: APS, antiphospholipid syndrome; β_2-GPI, β_2-glycoprotein I; Chol, cholesterol; CL, cardiolipin; DOPC, dioleoylphosphatidylcholine; DPPS, dipalmitoylphosphatidylserine; LDL, low-density lipoprotein; oxLDL, oxidized LDL.

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early reported to locate in domain V (15), and a tertiary structure of the PL binding cluster was most recently demonstrated (16). β_2-GPI also inhibits the intrinsic blood coagulation pathway (17), prothrombinase activity (18), and ADP-dependent platelet aggregation (19). We recently reported that β_2-GPI inhibits activated protein C activity (20).

Early atherosclerotic lesions characteristically contain neutral lipid-laden foam cells, which are, at least in part, derived from macrophages that have taken up cholesteryl ester-rich lipoproteins (21, 22). Oxidized low density lipoprotein (oxLDL) is bound and internalized by macrophages via scavenger receptors, which are not down-regulated, when the cholesterol content of the cell increases. There is increasing evidence supporting the oxidation of LDL as a significant element in the pathogenesis of atherosclerosis (23–26).

Vaarala et al. (27) reported that aCL raised in SLE patients might cross-react with malondialdehyde (MDA)-modified LDL. Later, others failed to show any cross-reaction between anti-β_2-GPI and anti-oxLDL antibodies in the APS patients (28). It was also shown that anti-β_2-GPI Abs could be a marker for arterial thrombosis in SLE patients, whereas IgG anti-oxLDL was not associated with arterial thrombosis (29). We found a specific interaction among $CuSO_4$-oxidized plasma lipoproteins, β_2-GPI, and anti-β_2-GPI Abs, and involvement of β_2-GPI and an anti-β_2-GPI Ab in oxLDL uptake by macrophages (30). However, the precise ligand on oxidized lipoproteins for β_2-GPI remained to be elucidated.

There was another set of reports suggesting that some aPL in APS patients are directed to neoepitopes of oxidized PLs, or those generated by adduct formation between oxidized PLs and associated proteins (31, 32), and that a candidate of oxidized PLs, i.e., 1-palmitoyl-2-(5-oxovaleroyl)-phosphatidylcholine (POVPC), which is one of the oxidized forms of 1-palmitoyl-2-arachidonoyl-phosphatidylcholine (PAPC), comes from oxidation of LDL (33, 34).

The major objective of our recent studies is to determine the involvement of β_2-GPI and anti-β_2-GPI auto-Abs in the development of atherosclerosis and/or atherothrombosis in APS patients. In the present study, interaction among oxLDL, β_2-GPI, and anti-β_2-GPI auto-Abs was analyzed, an oxLDL-derived ligand (oxLig-1) specific for β_2-GPI was isolated, and its structure was characterized. We also showed that liposomes containing oxLig-1, as a model of oxLDL, were avidly taken up by macrophages, by a process dependent on both β_2-GPI and anti-β_2-GPI Abs. Finally, anti-β_2-GPI auto-Abs derived from APS patients with episodes of arterial thrombosis were detected in enzyme-linked immunosorbent assay (ELISA), using a solid-phase oxLig-1 complexed with β_2-GPI.

MATERIALS AND METHODS

Chemicals

L-α-Dipalmitoylphosphatidylserine (DPPS), CL, and cholesterol (Chol), 7-ketocholesterol (5-cholesten-3β-ol-7-one) were obtained from Sigma Chemical Co. (St. Louis, MO); PAPC, and dioleoylphosphatidylcholine (DOPC) from Avanti Polar Lipids Inc. (Alabaster, AL); and L-3-phosphatidyl[*N*-methyl-^{3}H]choline, 1,2-dipalmitoyl ([^{3}H]DPPC) (80 Ci/mmol) from Amersham-Pharmacia Biotech (Uppsala, Sweden). All other chemicals were from commercial sources and of reagent-grade quality.

Preparation of human β_2-GPI

β_2-GPI was purified from normal human plasma as described (35) with slight modification. Pooled plasma from healthy subjects were chromatographed on a heparin-Sepharose column (or CL-polyacrylamide gel column), on a DEAE-cellulose column, and on an anti-β_2-GPI affinity column. To remove any contamination by IgGs, the β_2-GPI-rich fraction was further passed through a protein A-Sepharose column. The final β_2-GPI fraction was delipidated by extensive washing with *n*-butanol.

Patients' sera

Anti-β_2-GPI positive sera were obtained from APS patients with episodes of arterial thrombosis. Informed consent was given for all patients and the study was approved by the Institutional Review Board of Okayama University Medical School (Okayama, Japan). To eliminate endogenous β_2-GPI for some experiments, sera were passed through a heparin-Sepharose column. The effluent was dialyzed against PBS and was used for ELISAs.

Monoclonal antibodies (mAbs)

A human monoclonal anti-β_2-GPI auto-Ab, EY2C9 (IgM), was established from peripheral blood lymphocytes from an APS patient (36). A mouse monoclonal anti-β_2-GPI auto-Ab, WB-CAL-1 (IgG2a, κ), was derived from an (NZW x BXSB) F1 mouse (37). The monoclonal anti-human β_2-GPI Ab, Cof-22 (IgG1, κ), was established from BALB/c mice immunized with human β_2-GPI (38). Both EY2C9 and WB-CAL-1 mAbs bind either to a complex of β_2-GPI and CL or to β_2-GPI adsorbed on an oxygenated polystyrene plate. In contrast, Cof-22 is specific for human β_2-GPI and recognizes its native structure.

Preparation of oxLDL and lipid extraction

Plasma LDL ($1.019 < d < 1.063$) was isolated by ultracentrifugation from fresh normal human plasma, as described (39). LDL (100 μg protein/ml) was oxidized by incubating with 5 μM $CuSO_4$ for 8 h at 37°C. To stop the oxidation, 1 mM EDTA was added and the oxidized sample was dialyzed extensively against PBS containing 1 mM EDTA. Protein concentration was determined using BCA protein assay reagent (Pierce Chemical Co., Rockford, IL). An aliquot was taken to determine thiobarbituric acid reactive substance (TBARS) value (40), and for use in agarose gel electrophoresis. A lipid fraction was extracted from LDLs according to the method of Folch, Lees, and Sloane-Stanley(41).

Assay for molecular interaction

Real-time molecular analysis was performed with an optical-biosensor, IAsys (Affinity Sensors, Cambridge, UK). β_2-GPI binding to LDLs or liposomes: Biotinyl-β_2-GPI was immobilized on a biotinyl-cuvette via streptavidin. LDLs or liposomes at various concentrations were placed in the cuvette. Ab binding to LDLs or liposomes: The biotinyl-WB-CAL-1 was immobilized on the cuvette. LDLs or liposomes were added at various concentrations in the presence (10 μg/ml) or absence of β_2-GPI.

ELISA for detecting β_2-GPI and anti-β_2-GPI Ab binding

Binding to LDLs. A microtiter plate (Immulon 2HB, Dynex Technologies Inc., Chantilly, VA) was coated with 50 μl of $F(ab')^2$ of anti-apoB 100 Ab, 1D2 (10 μg/ml, Yamasa Corp., Choshi, Japan) by incubation overnight at 4°C. After blocking with PBS containing 1% skim milk, the plate was incubated with LDLs for 1 h. The wells were incubated sequentially with β_2-GPI (15 μg/ml), Cof-22, and horseradish peroxidase (HRP)-labeled anti-mouse IgG, each for 1 h. The color was developed with H_2O_2 and *o*-phenylenediamine, and absorbance was measured at 490 nm.

Binding to extracted lipids. A microtiter plate (Immulon 1B, Dynex Technologies Inc.) was coated with lipids extracted from LDLs (50 μg/ml, 50 μl/well) by ethanol evaporation. The wells were blocked with PBS containing 1% BSA for 1 h and the wells were incubated with 30 μg/ml of β_2-GPI for 1 h. Then, β_2-GPI binding was detected using Cof-22 and HRP-labeled anti-mouse IgG. Alternatively, the coated plate was treated with β_2-GPI in the presence of anti-β_2-GPI auto-Abs (WB-CAL-1/ or EY2C9), and then with HRP-labeled anti-mouse IgG or anti-human IgM, respectively. The color was developed with H_2O_2 and *o*-phenylenediamine, and absorbance was measured at 490 nm. Between each step, extensive washing was done using PBS containing 0.05% Tween 20.

Thin-layer chromatography (TLC) and ligand blot analysis

Extracted lipids were spotted on a Polygram silica gel plate (Machery-Nagel, Duren, Germany) and developed in chloroform/methanol/30% ammonia/water (120:80:10:5, v/v/v/v, solvent A). Plates were stained with I_2 vapor, or with a spray of molybdenum blue, of 2 N sulfuric acid containing 2% orcin, or of glacial acetic acid/sulfuric acid (19:1, v/v) (the Lieberman-Burchard reaction). Alternatively, the developed plate was subjected to ligand blot with β_2-GPI and an anti-β_2-GPI Ab. Thin-layer chromotography (TLC) plates were blocked with PBS containing 1% bovine serum albumin (BSA) and were subsequently incubated with β_2-GPI, anti-β_2-GPI Abs (Cof-22, WB-CAL-1, or EY2C9), and HRP-labeled anti-mouse IgG or anti-human IgM each for 1 h. In each step, plates were extensively washed with PBS. The color was developed with H_2O_2 and 4-methoxy-1-naphtol (Aldrich, Milwaukee, WI). The ligand-enriched bands scraped from the TLC plate were subjected to another TLC in chloroform/methanol (8:1, v/v) (solvent B). For large-scale purification of the ligand, extracted lipids were loaded on a TLC silica gel 60 plate (PLC plate; Merck, Darmstadt, Germany) of 2-mm thickness.

High performance liquid chromatography (HPLC)

A ligand-enriched preparation from oxLDL obtained by two-step TLC was analyzed by reversed-phase HPLC on a Sephasil Peptide C18 5-μm column (250 × 4.6 mm; Amersham-Pharmacia Biotech). The column was eluted with a mixture of acetonitrile/isopropanol/water (60:30:2, v/v/v, solvent C) at a flow rate of 0.5 ml/min and fractionated every minute. Each eluate was spotted on a TLC plate and subjected to ligand blot with β_2-GPI and EY2C9.

Mass spectroscopy and nuclear magnetic resonance (NMR)

oxLig-1 was analyzed on a Shim-pack VP-ODS column (150 × 4.6 mm), with a LC/MS-QP8000α (Shimadzu Corp., Kyoto, Japan), using solvent C (liquid chromatography equipped mass spectroscopy, LC/MS). Positive and negative ionization mass spectra were taken in the mass range of 50–850. The field desorption (FD) mass spectra of synthesized oxLig-1 and methylated oxLig-1 were recorded on JMS-SX102A and JMS AX-500 (JEOL, Tokyo, Japan), respectively. ^{1}H-NMR and ^{13}C-NMR spectra were measured at 300 and 75.5 MHz, respectively, on ASX-300 spectrometer (Bruker, Billerica, MA).

Preparation of 7-ketochoresteryl-9-caboxynonanoate (synthesized oxLig-1, syn-oxLig-1)

To a solution of 7-ketocholesterol (5-cholesten-3β-ol-7-one, 50.1 mg, 0.125 mmol) and azelaic acid (70.6 mg, 0.375 mmol) in acetone (4 ml) was added 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (WSC; 95.8 mg, 0.500 mmol) and 4-(dimethylamino) pyridine (DMAP; 30.5 mg, 0.25 mmol) (Fig. 6). The mixture was stirred at room temperature for 2 days, concentrated, and extracted with chloroform. The extract was successively washed with 2 M hydrochloric acid and brine, dried over anhydrous magnesium sulfate, and evaporated. The residue was subjected to column chromatography on silica gel using toluene/ethyl acetate (3:1, v/v) to give syn-oxLig-1 (36.0 mg, 50.4% yield). ^{1}H-NMR of syn-oxLig-1 (300 MHz, $CDCl_3$): δ = 5.71 (s, ^{1}H, H-6), 4.78-4.69 (m, ^{1}H, H-3); ^{13}C-NMR (75.5 MHz, $CDCl_3$): δ = 202.5, 179.7, 173.4, 164.5, 127.1, 72.4, 55.2, 50.4, 50.2, 45.8, 43.5, 39.9, 38.7, 36.6, 36.1, 29.2, 28.9, 28.4, 25.3, 25.0, 24.2, 23.2, 23.0, 19.3, 17.7, 12.4.; *m/z* (FD-MS): 571 [$(M+H)^+$, $C_{36}H_{59}O_5$ requires 571] (Fig. 7).

Methylation of oxLig-1

To a solution of purified and/or synthesized oxLig-1 (7.5 mg, 0.0131 mmol) in ether (2 ml) was added diazomethane-ether solution at room temperature, and the mixture was stirred for 2 h. After addition of acetic acid to the stirring solution for 1 day, the mixture was evaporated to give methylated oxLig-1 preparation as a single product. ^{1}H-NMR of methylated syn-oxLig-1 (300 MHz, $CDCl_3$): δ = 5.71 (s, ^{1}H, H-6), 4.78-4.69 (m, m, ^{1}H, H-3), 3.67 (s, ^{1}H, COOCH3); ^{13}C-NMR (75.5 MHz, CDCl3): δ = 202.4, 174.7, 173.4, 164.4, 127.1, 72.4, 55.2, 50.4, 45.8, 43.5, 39.9, 38.7, 36.6, 36.4, 36.1, 29.3, 28.4, 26.7, 25.3, 24.2, 23.2, 23.0, 21.6, 19.3, 17.7, 12.4.; *m/z* (FD-MS): 585 [$(M+H)^+$, $C_{37}H_{61}O_5$ requires 585].

Preparation of liposomes

Liposomes were prepared as described (42), with the following lipid compositions. Lipid molar ratios of 0, 10, 25, and 50% PS-liposomes are as follows: DOPC/DPPS/[^{3}H]DPPC (80 Ci/mmol) (100/0/0.00225, 90/10/0.00225, 75/25/0.00225, and 50/50/0.00225). Lipid molar ratios of 0, 5, 12.5, and 25% PS-Chol-liposomes are as follows: DOPC/DPPS/Chol/[^{3}H]DPPC (50/0/50/0.00225, 45/5/50/0.00225, 37.5/12.5/50/0.00225, and 25/25/50/0.00225); 0, 10, 20, and 40% oxLig-1-liposomes: DOPC/oxLig-1/[^{3}H]DPPC (100/0/0.00225, 90/10/0.00225, 80/20/0.00225, and 60/40/0.00225); 0, 5, 10, 20, 30, and 40% oxLig-1-Chol-liposomes: DOPC/Chol/oxLig-1/[^{3}H]DPPC (50/50/0/0.00225, 50/45/5/0.00225, 50/40/10/0.00225, 50/30/20/0.00225, 50/20/30/0.00225, and 50/10/40/0.00225). Syn-oxLig-1-liposomes were prepared using syn-oxLig-1 as for oxLig-1-liposomes. A mixture of the desired lipids in chloroform/methanol (1:1, v/v) was placed in a pear-shaped flask and the solvent was removed in a rotary evaporator under reduced pressure. The dried lipids were dispersed with a vortex mixer in 0.3 M glucose solution. Then the liposome solution was sonicated for 5 min at 70°C with a probe-type sonicator.

Cell culture and liposome binding

A monolayer culture of murine macrophage-like cells, J774A.1, obtained from Riken Cell Bank (Tsukuba, Japan) was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). For binding experiments, the cells (8×10^5 cells/ml) were dispensed 1 ml/well into a 12-well culture plate (Sumitomo Bakelite Co., Ltd. Tokyo, Japan) and incubated for 24 h at 37°C; then the culture medium was replaced with Celgrosser-P medium (Sumitomo Pharmaceutical Co., Tokyo, Japan). After 1 h of preincubation at 37°C, 50 μl of liposomes (50 nmol lipid/well) with or without β_2-GPI (200 μg/ml) and WB-CAL-1 (100 μg/ml) were added to each culture, and the cells were incubated at 4°C and/or 37°C. The wells were next washed with chilled PBS, and the cells were lysed by adding 1 ml of 0.1 N NaOH. An aliquot was taken to determine cellular proteins and radioactivity associated with the cells. Protein concentration was determined using the BCA protein assay reagent (Pierce).

Anti-β_2-GPI ELISA

Anti-β_2-GPI ELISA was performed as described (43). Briefly, β_2-GPI was adsorbed on an oxygenated polystyrene plate (carboxylated, Sumilon C-type, Sumitomo Bakelite Co., Ltd.) by in-

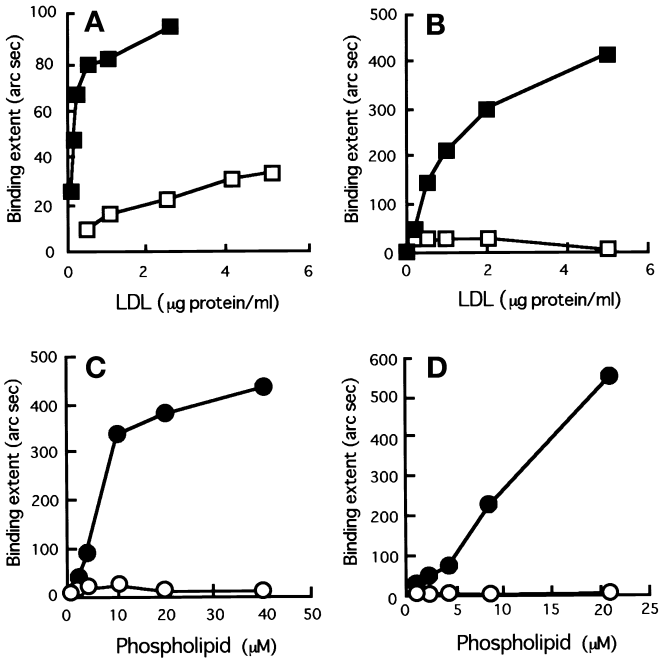

Fig. 1. Molecular interactions among β_2-GPI, LDLs, phospholipids (PLs), and anti-β_2-GPI auto-Ab, detected by an optical biosensor. A: LDL (□) or oxLDL (■) binding to solid phase β_2-GPI. B: LDL (□) or oxLDL (■) binding to solid phase WB-CAL-1 in the presence of β_2-GPI. C: DOPE (○) or CL (●) binding to solid phase β_2-GPI. D: DOPE (○) or CL (●) binding to solid phase WB-CAL-1 in the presence of β_2-GPI.

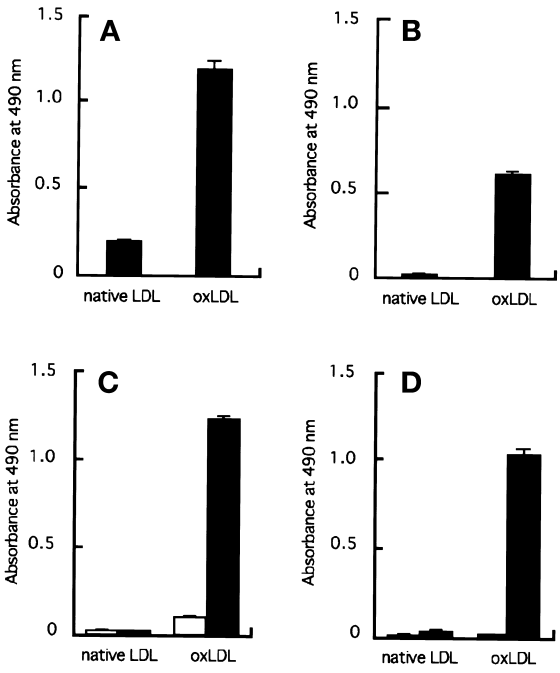

Fig. 2. Binding of β_2-GPI and anti-β_2-GPI auto-Abs to plasma LDLs or their lipid extracts. A: Plasma LDLs, β_2-GPI, and mouse anti-human β_2-GPI mAb, Cof-22, were sequentially incubated in a plate coated with Fab fragment of anti-apoB100 mAb, 1D2. Binding was detected using HRP-labeled anti-mouse IgG. B–D: Lipid extracts from LDLs were coated on a plate. β_2-GPI binding was detected using Cof-22 and using HRP-labeled anti-mouse IgG (B). Subsequent binding of WB-CAL-1 (C) and EY2C9 (D) were detected using HRP-labeled anti-mouse IgG (C) and with HRP-labeled anti-human IgM (D), respectively. Open columns: without β_2-GPI, closed columns: with β_2-GPI (C, D). Data are indicated as the mean ± SD of triplicate samples.

cubating at 10 μg/ml (50 μl/well). The plates were blocked with 3% gelatin and 100 μl/well of anti-β_2-GPI mAb or of 100-fold-diluted serum samples were applied for 1 h. Ab binding to β_2-GPI was probed using HRP-labeled anti-human IgG or IgM or anti-mouse IgG. The color was developed with H_2O_2 and *o*-phenylenediamine, and absorbance was measured at 490 nm. Between steps, extensive washing was done using PBS containing 0.05% Tween 20.

ELISA for Abs against a protein-oxLig-1 complex

Syn-ox-Lig-1 (50 μg/ml, 50 μl/well) was adsorbed by evaporation on a plain polystyrene plate (Immulon 1B), and the plate was then blocked with 1% BSA. Sera (diluted 1:100 with PBS containing 0.3% BSA) were incubated in the wells with β_2-GPI (15 μg/ml) or other proteins for 1 h at room temperature. Ab binding was detected with HRP-labeled anti-human IgG. Further steps were performed as described in "anti-β_2-GPI ELISA."

ELISA for β_2-GPI-dependent aCL

CL (50 μg/ml, 50 μl/well) was adsorbed on a plain plate (Immulon 1B), and further steps were performed as described in "ELISA for Abs against a protein-oxLig-1 complex."

RESULTS

Molecular interaction

oxLDL, but not native LDL, showed highly specific binding to β_2-GPI (**Fig. 1A**). A dose-dependent binding to solid phase WB-CAL-1 of oxLDL was observed only in the presence of β_2-GPI (10 μg/ml), and no specific binding of native LDL was observed (Fig. 1B). In a control experiment, CL showed a large extent of binding, whereas dioleoylphosphatidylethanolamine (DOPE) did not show any specific binding (Fig. 1C). CL also showed dose-dependent binding to WB-CAL-1 in the presence of β_2-GPI, whereas DOPE did not (Fig. 1D).

Binding of β_2-GPI and anti-β_2-GPI Ab to solid-phase LDLs or derived lipids

β_2-GPI specifically bound with high avidity to immobilized oxLDL but minimally to native LDL (**Fig. 2A**). Lipids were extracted from LDLs, immobilized on a plate, and subjected to binding assays for β_2-GPI, by detecting with Cof-22 mAb and anti-β_2-GPI Abs (i.e., WB-CAL-1 and EY2C9). The assays showed specific binding of β_2-GPI (Fig. 2B) and of β_2-GPI-mediated Ab-binding to the lipids from oxLDL (Fig. 2C and D) but did not to those from native LDL (Fig. 2B–D).

Purification and characterization of a β_2-GPI-specific ligand

The lipids extracted from both LDLs were spotted on a TLC plate and developed in solvent A (**Fig. 3A** and **B**). In the plates treated with I_2 vapor and molybdenum blue, decreased PC and increased polar forms co-migrating with

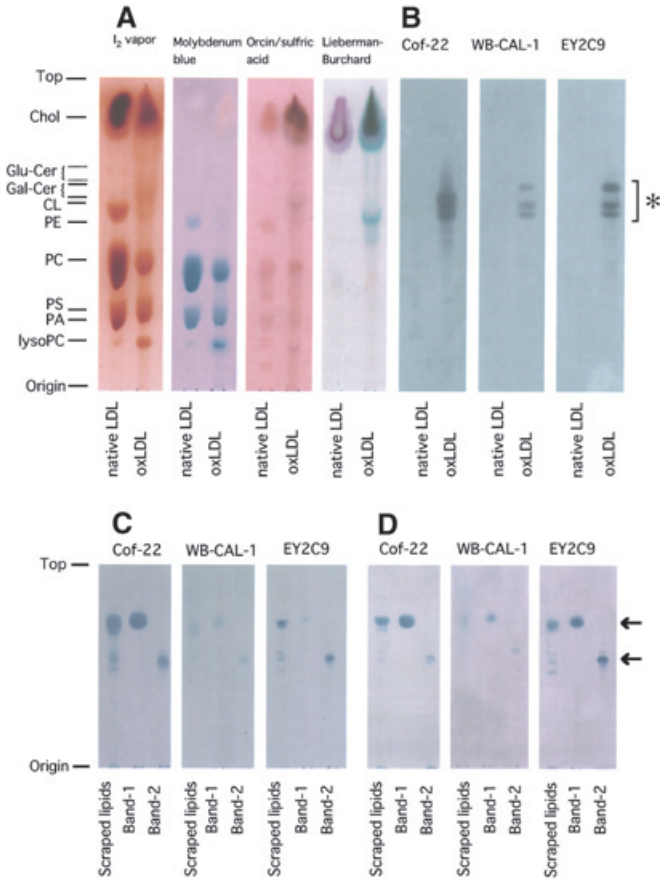

Fig. 3. Thin-layer chromatography (TLC) and ligand blot of lipid extracts from LDLs. Lipids extracted from LDLs were spotted on silica gel plates and developed in solvent A. A: Staining with I_2 vapor, molybdenum blue, orcin/sulfuric acid, and sulfuric acid/acetic acid as indicated in the figure. B: Ligand blot was performed with β_2-GPI and anti-β_2-GPI Abs. The region marked with an asterisk was scraped off and subjected to further purification. Glu-Cer, glucosylceramide; Gal-Cer, galactosylceramide. C: Ligand blot of the scraped lipids, Band-1 and Band-2, was performed by sequential treatment with β_2-GPI and anti-β_2-GPI Abs (2-step). D: Ligand blot of the eluate was performed by co-incubation of β_2-GPI and anti-β_2-GPI Abs (1-step).

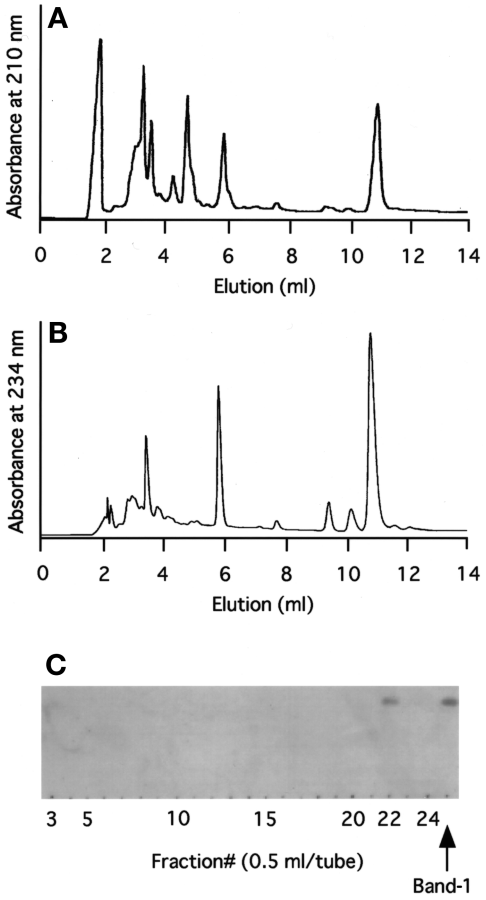

Fig. 4. Elution profiles of Band-1 by reversed-phase high performance liquid chromatography (HPLC). Scraped Band-1 was eluted on Sephacil-Peptide column, detected at 210 nm (A) and 234 nm (B). Data of ligand blot analysis on eluate using EY2C9 are also shown in (C).

lysoPC were observed in lipids extracted from oxLDL as compared with those of native LDL. By staining with orcin/sulfuric acid, pseudo-positive bands were visible at similar Rf positions of Chol, PC, CL, and others. In the Lieberman-Burchard reaction, a Chol band was visible for both extracts almost at the top, and a few bands near the Rf position of CL were observed only for lipid extract from oxLDL. To identify β_2-GPI-specific ligands, the developed plates were subjected to ligand blot in which the plates were subsequently treated with β_2-GPI and anti-β_2-GPI Abs (Fig. 3B). With all three tested anti-β_2-GPI Abs, Cof-22, WB-CAL-1, and EY2C9, bands of oxLDL lipids, which had similar Rf values to that of CL, were visualized.

β_2-GPI-ligand-enriched lipids were scraped from the first TLC plate ("scraped lipids") (Fig. 3B) and were subjected to another TLC, developing in solvent B, and two major bands were scraped off and subjected to the ligand blot (Fig. 3C and D). Two major bands (indicated by arrows) reacting with β_2-GPI were named Band-1 (the upper band) and Band-2 (the lower band). Band-1 was rarely stained in the sequential treatment with β_2-GPI and EY2C9/ and WB-CAL-1, but was strongly stained in the simultaneous treatment. In contrast, Band-2 stained well in either treatment with β_2-GPI and EY2C9 (densitometric analysis in individual experiments indicated significant differences). The alkaline hydrolyzed (20% NaOH, 100°C, 30 min) product of Band-1 did not bind to β_2-GPI in the ligand blot (data not shown). Band-1 was subjected to reversed-phase HPLC in solvent C. A major peak appeared at 22 min that positively stained in the ligand blot with β_2-GPI and EY2C9 (**Fig. 4**), and produced a very weak Lieberman-Burchard reaction. This peak was designated as oxLig-1. From 100-mg protein equivalent of oxLDL, approximately 2.5 mg of oxLig-1 was recovered.

oxLig-1 was further analyzed by LC/MS. The intensities of both positive and negative ions from oxLig-1 were detected for the main peak (at 8.3 min) at 234 nm (**Fig. 5A**, upper). A positive ionization mass spectrum gave a signal at *m/z* 571, which was considered to be the $(M + H)^+$ ion, and at *m/z* 383 (Fig. 5D), which was identical to 7-ketocholesterol ion (Fig. 5B). In the negative ion mode, a signal of fragment ion at *m/z* 187 was detected (Fig. 5C). We treated oxLig-1

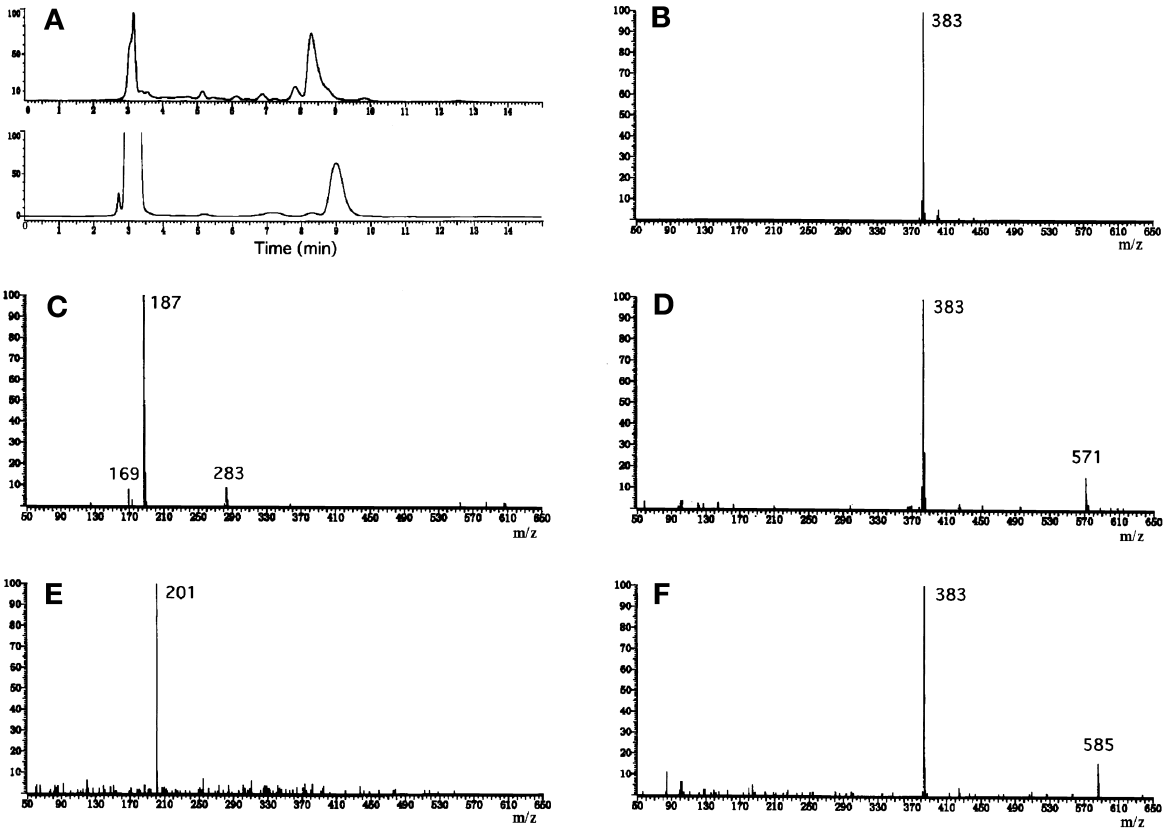

Fig. 5. LC/MS of purified oxLig-1 and its methylated compound. A: HPLC profiles of oxLig-1 (upper) and methylated oxLig-1 (lower) at 234 nm. B: A positive ionization mass spectrum of 7-ketocholesterol. C: A negative ionization mass spectrum of oxLig-1. D: A positive ionization mass spectrum of oxLig-1. E: A negative ionization mass spectrum of methylated oxLig-1. F: A positive ionization mass spectrum of methylated oxLig-1.

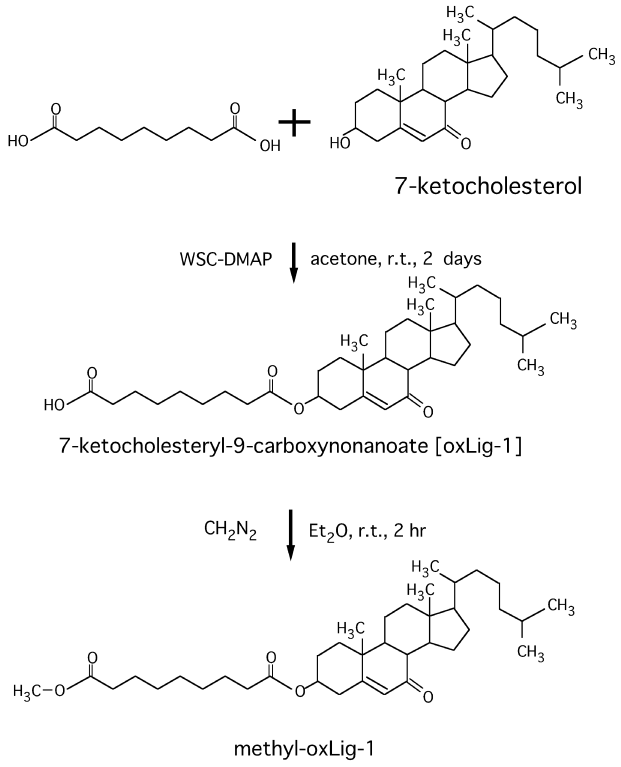

Fig. 6. Synthesis of oxLig-1 (7-ketocholesteryl-9-carboxynonanoate) and its methylation.

with diazomethane in diethyl ether to give methylated oxLig-1, and also analyzed it by LC/MS. Methylated oxLig-1 was eluted later than oxLig-1 (Fig. 5A, lower), and signals of the largest fragment ion (9.0 min) were detected at *m/z* 585 and m/z 201, in the positive and negative ion modes, respectively (Fig. 5E and F). These data are consistent with the structure of 7-ketocholesteryl-9-carboxynonanoate.

Synthesis and analysis of oxLig-1

To confirm the structure of oxLig-1, we synthesized 7-ketocholesteryl-9-carboxynonanoate from 7-ketocholesterol and azelaic acid. As shown in **Fig. 6**, both materials were conjugated with WSC and DMAP in acetone at room temperature for 2 days. The product was isolated by column chromatography on silica gel. The structure of synthetic oxLig-1 was verified to purified oxLig-1 by ^{1}H- and ^{13}C-NMR spectroscopy and FD mass spectrometry. In a ^{1}H-NMR spectrum of synthesized oxLig-1 (syn-oxLig-1) (**Fig. 7A**), the signals of H-3 and H-6 are shown at δ 4.69–4.78 and 5.71 ppm, as multiplet and singlet, respectively. Furthermore, three peaks assignable to carbonyl carbons are shown at δ 202.5, 179.7, and 173.4 ppm together with two signals of olefin carbons at δ 164.5 and 127.1 ppm (Fig. 7B). The syn-oxLig-1 was then esterified with diazomethane in diethyl ether to give methylated oxLig-1. In Fig. 7C and D, ^{1}H- and ^{13}C-NMR

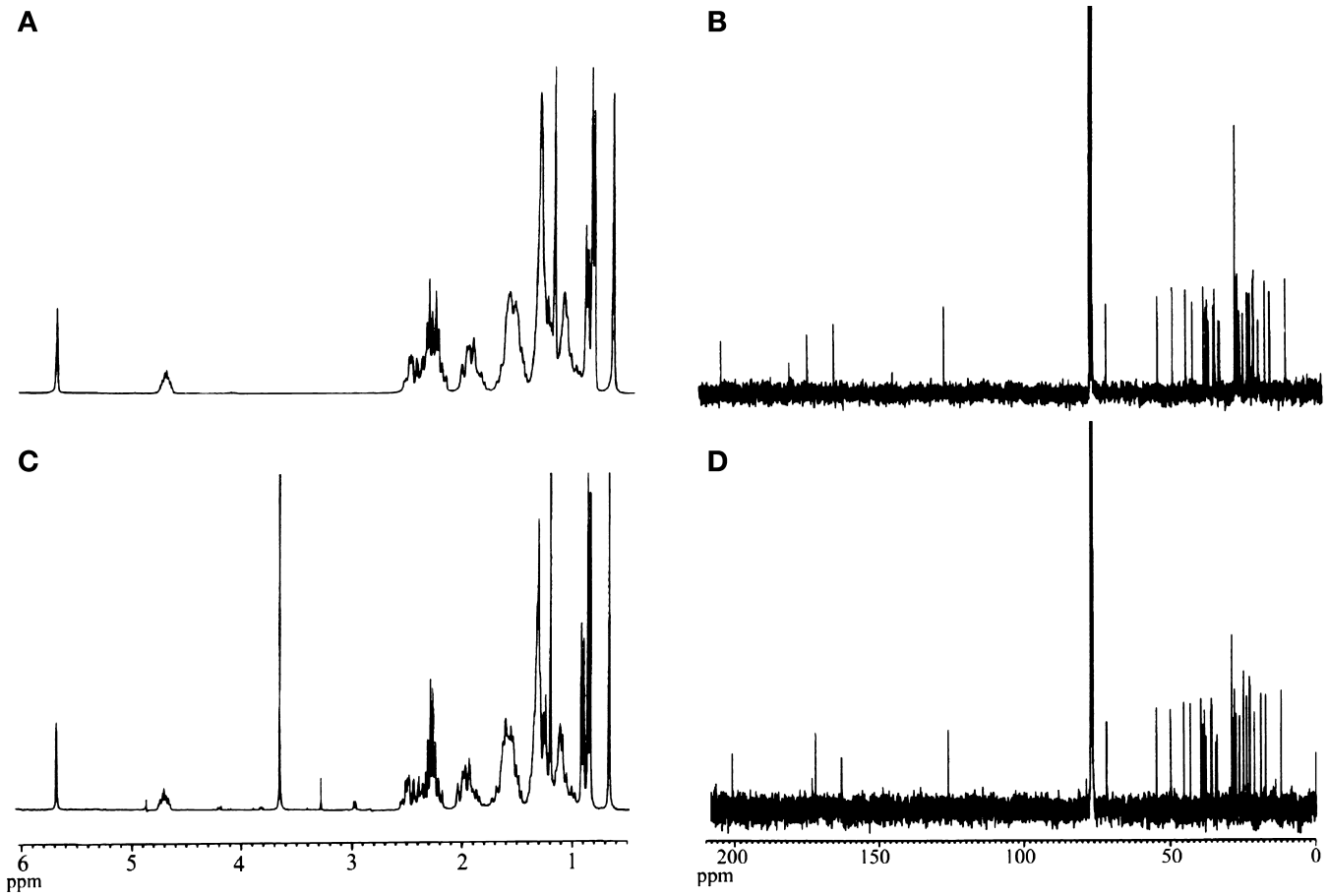

Fig. 7. Nuclear magnetic resonance (NMR) of syn-oxLig-1 and its methylated compound. A 300-MHz ^{1}H-NMR spectrum of syn-oxLig-1 (A) or of methylated oxLig-1 (C). 75.3-MHz ^{13}C-NMR of syn-oxLig-1 (B) or of methylated syn-oxLig-1 (D).

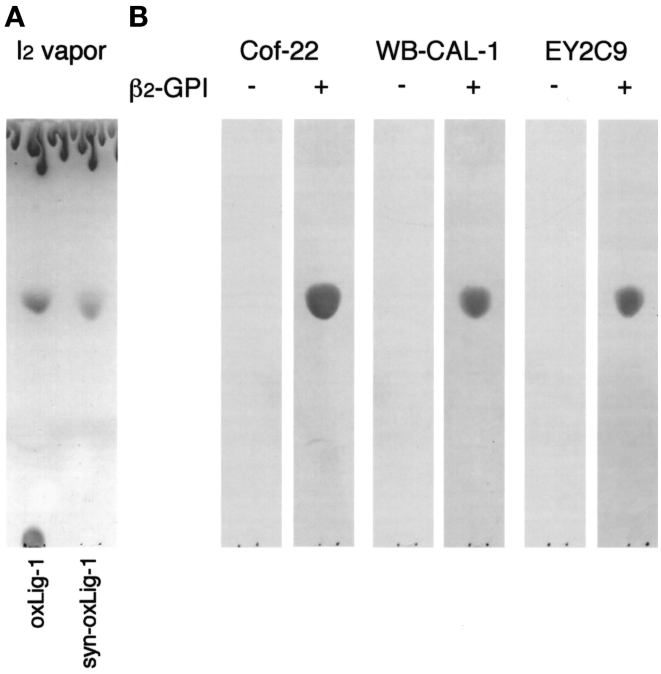

Fig. 8. TLC and ligand blot analysis on syn-oxLig-1. A: oxLig-1 and syn-oxLig-1 was spotted on a TLC plate, developed with solvent A, and detected with I_2-vapor. B: Ligand blot of syn-oxLig-1 was performed with anti-β_2-GPI Abs in the presence (+) or absence (−) of β_2-GPI.

spectra of methylated syn-oxLig-1 are shown. The new singlet observed in its ^{1}H-NMR spectrum at δ 3.67 ppm strongly suggested that oxLig-1 had carboxyl function (Fig. 7C). Then, syn-ox-Lig-1 was subjected to TLC and ligand blot analysis with β_2-GPI and anti-β_2-GPI Abs. Syn-oxLig-1 showed the same Rf position and binding characters to Cof-22, WB-CAL-1, and EY2C9 Abs, as previously described for oxLig-1 derived from oxLDL (Fig. 3 and **Fig. 8**). The syn-oxLig-1 and its methylated compound were further analyzed by LC/MS. The LC chromatograms and mass spectra of both compounds (**Fig. 9**) were identical to counterparts from oxLDL (Fig. 5). In Fig. 9D, the small peak at 8.3 min contains syn-oxLig-1 that remained underivatized after the methylation reaction. The underivatized material was identified as syn-oxLig-1 by mass spectra.

Liposome binding to macrophages

Binding to the J774A.1 cells of exogenous PS-Chol-liposomes increased depending on the amount of DPPS (**Fig. 10**). In contrast, binding to the cells of oxLig-1-Chol-liposomes was relatively low. Similar binding profiles were obtained with Chol-free liposomes of PS or oxLig-1. When mouse peritoneal macrophages were used in place of J774A.1 cells, comparable liposome binding was observed.

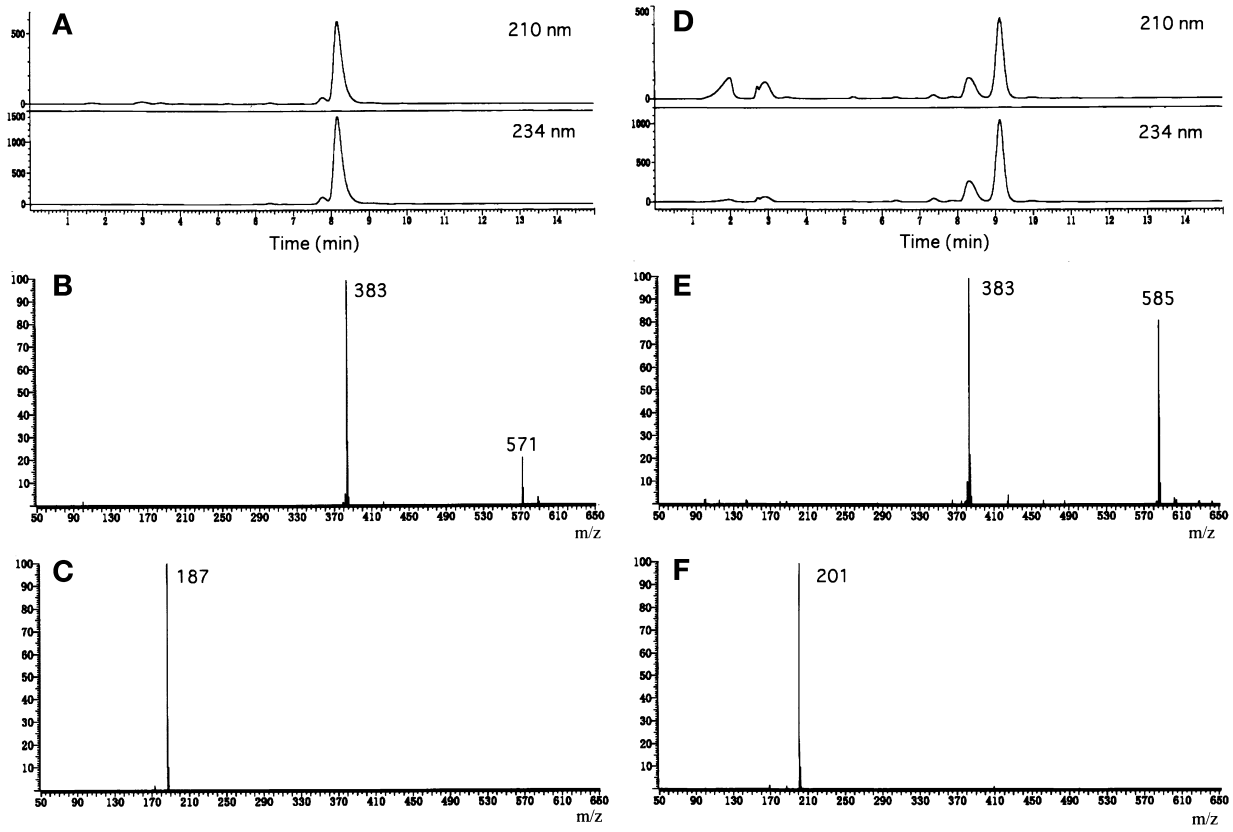

Fig. 9. LC/MS of syn-oxLig-1 and its methylated compound. A: LC chromatograms of syn-oxLig-1 at 210 and 234 nm. B: A positive ionization mass spectrum of syn-oxLig-1. C: A negative ionization mass spectrum of syn-oxLig-1. D: LC chromatograms of methylated syn-oxLig-1 at 210 and 234 nm. E: A positive ionization mass spectrum of methylated syn-oxLig-1. F: A negative ionization mass spectra of methylated syn-oxLig-1.

Ab-dependent liposome binding to macrophages

The binding (4°C, 2 h) of PS-liposomes and oxLig-1-liposomes increased dramatically upon simultaneous addition of β_2-GPI and WB-CAL-1, and was also dependent on the concentration of WB-CAL-1 (**Fig. 11 A** and **B**). In the same assay, subclass-matched control Abs had no effect on such binding. The uptake (37°C, 5 h) of oxLig-1-liposomes by J774A.1 cells increased significantly (4.36 pmol [^{3}H]DPPC/mg protein) by incubating with β_2-GPI and WB-CAL-1, as compared with incubation without β_2-GPI and WB-CAL-1 (0.72 pmol [^{3}H]DPPC/mg protein) (data not shown). As shown in Fig. 11C, binding of syn-oxLig-1-liposomes to the macrophages also increased depending on the ligand concentration in liposomes. In the case of syn-oxLig-1, the binding almost reached a plateau at the concentration of 10 mol%.

Detection of auto-Abs in APS patients with episodes of arterial thrombosis in ELISA using a β_2-GPI-syn-oxLig-1 complex

As shown in **Fig. 12**, auto-Abs against a complex of β_2-GPI and syn-oxLig-1 were frequently detected in APS patients. There was a good correlation between values of auto-Abs against the complex antigen and those of β_2-GPI-dependent aCL and anti-β_2-GPI Abs. As far as we tested, such auto-Abs derived from APS cross-reacted with β_2-GPI complexed with CL and oxLig-1, but not that complexed with oxidized PAPC (oxPAPC) (**Table 1**, Exp. 1). The Ab binding did not correlate with the amount of TBARS of lipid. Further, the Ab binding was provided only by the interaction between oxLig-1 and intact β_2-GPI (Exp. 2). In contrast, no Ab binding was provided by nicked β_2-GPI or haptoglobin having "Sushi domains" that did not have PL binding properties.

DISCUSSION

In the present study, we obtained strong evidence for specific binding interactions among β_2-GPI, oxLDL, and an anti-β_2-GPI auto-Ab, using an optical biosensor. We purified a ligand specific for β_2-GPI and characterized its structure and involvement in macrophage uptake of oxLDL using a synthesized ligand. The structure of the ligand was confirmed by reproducing its properties with chemically synthesized 7-ketocholesteryl-9-carboxynonanoate.

It appears that oxidation of LDL plays an important role in atherogenesis (44). To examine mechanisms related to the development of atherosclerosis, several experimental models of denatured LDL, such as MDA-LDL, acetylated LDL, and $CuSO_4$-mediated oxLDL ($CuSO_4$-oxLDL), have been used. Among these LDLs, trace amounts of Cu^{2+}-ion can induce LDL oxidation, resulting in highly reproducible LDL damage (45). This process leads to

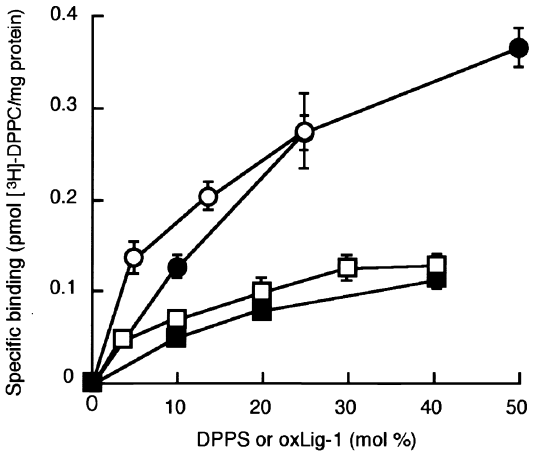

Fig. 10. Effect of phosphatidylserine (PS) or oxLig-1 content on the binding of liposomes to macrophages. A monolayer of J774.A1 cells was incubated for 2 h at 4°C with Celgrosser-P medium containing ^{3}H-labeled liposomes (50 nmol lipid/well). (○) PS-liposomes; (●) PS-Chol-liposomes; (□) oxLig-1-liposomes; (■) oxLig-1-Chol-liposomes. Data are indicated as the mean ± SD of triplicate samples.

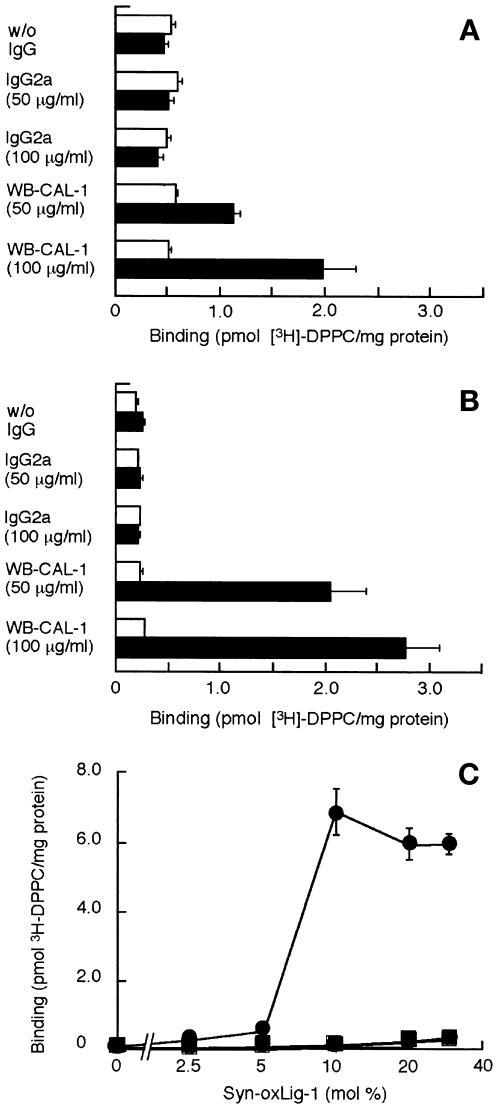

Fig. 11. β_2-GPI and anti-β_2-GPI mAb-dependent binding of ligand-containing liposomes to macrophage. A monolayer of J774.A1 cells was incubated for 2 h at 4°C with Celgrosser-P medium containing ^{3}H-labeled liposomes (50 nmol lipid/well) and WB-CAL-1, in the presence or absence of β_2-GPI (200 μg/ml). A: Binding of PS-liposomes (PS: 50 mol%) to J774.A1 cells in the presence (■) or absence (□) of β_2-GPI (200 μg/ml). B: Binding of oxLig-1-liposomes (oxLig-1: 40 mol%) to J774.A1 cells in the presence (■) or absence (□) of β_2-GPI (200 μg/ml). C: A monolayer of J774.A1 cells was incubated for 2 h at 4°C with Celgrosser-P medium containing ^{3}H-labeled syn-oxLig-1-liposomes (50 nmol lipid/well) and WB-CAL-1, in the presence or absence of β_2-GPI (200 μg/ml). Binding of syn-oxLig-1-liposomes in the absence of β_2-GPI and WB-CAL-1 (○), binding of syn-oxLig-1-liposomes in the presence of β_2-GPI (□), binding of syn-oxLig-1-liposomes in the presence of WB-CAL-1 (■), binding of syn-oxLig-1-liposomes in the presence of both β_2-GPI and WB-CAL-1 (●). Data are indicated as the mean ± SD of triplicate samples.

oxLDL that shares many structural and functional properties with LDL oxidized by cells or LDL extracted from arterial atherosclerotic plaques. Incubation of LDL with any of several different types of cells, or with Cu^{2+}-ion even in the absence of cells, results in generating oxidatively modified LDL with similar properties (46). There is general agreement about the use of $CuSO_4$-oxLDL as an autoantigen, because oxLDL has been found in atheromatous lesions and oxLDL extracted from atherosclerotic lesions exhibits nearly all of the physicochemical and immunological properties of $CuSO_4$-oxLDL (24).

Abs against oxLDL recognize substances in atherosclerotic lesions that are not present in normal arteries. It was reported that aCL raised in SLE patients cross-reacted with MDA-LDL (27), while other research groups found that another population of anti-oxLDL Abs reacted to oxidized PC (such as POVPC)-protein adducts in LDL particles (33, 47). However, in recent studies, we found that TBARS generation was not consistent with β_2-GPI binding to $CuSO_4$-oxLDL (48). As MDA is a hydrophilic short-chain aldehyde, it readily diffuses away from LDL particles (49). After dialysis, we also observed that TBARS in the oxLDL preparations decreased to undetectably low levels.

In this study, $CuSO_4$-oxLDL showed highly specific binding to immobilized β_2-GPI (Fig. 1A). The weak binding of native LDL to β_2-GPI might reflect the binding of β_2-GPI to lipoprotein[a], which is composed of LDL and apolipoprotein[a] (apo[a]), as described (50). However, such interaction between native LDL and β_2-GPI may not expose suitable epitopes to anti-β_2-GPI auto-Ab (Fig. 1B). Further, highly specific binding of anti-β_2-GPI auto-Ab, two mAbs, and Abs in two typical anti-β_2-GPI positive sera (from APS patients with episodes of arterial thrombosis) was observed only with the complex of β_2-GPI and the lipid ligand derived from $CuSO_4$-oxLDL (Figs. 2, 3, and 12; Table 1). Oxidative modification of LDL actually includes a series of complex changes such as lipid peroxidation, modification of side chains of amino acids by active aldehydes, increased surface charge, and polymerization. Among diverse modified molecules in oxLDL, β_2-GPI obviously bound to a component in the lipid moiety.

Linoleic acid is a predominant polyunsaturated fatty acid in LDL and is present mainly as Chol-ester (51). In

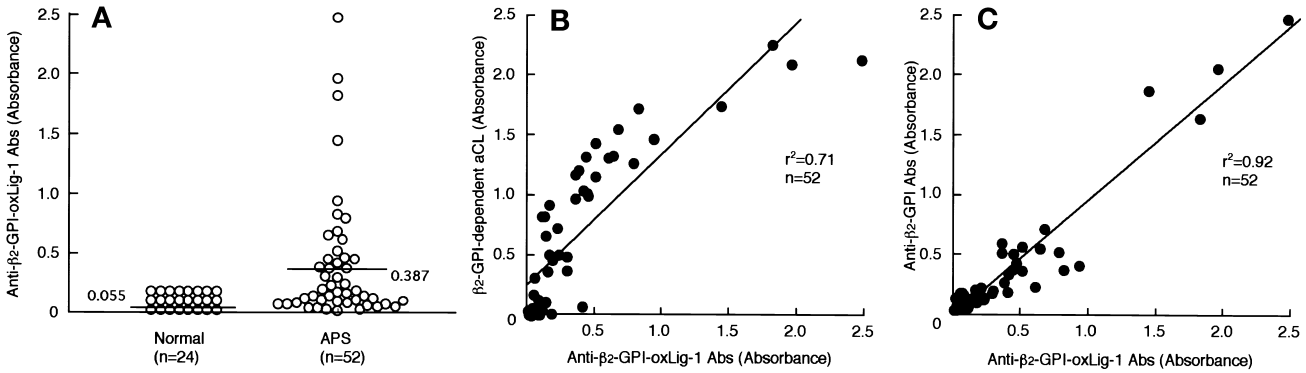

Fig. 12. Auto-Abs in APS sera against solid-phase β_2-GPI-oxLig-1 complex. Sera were obtained from normal subjects (n = 24) and APS patients with episodes of thrombosis (n = 52). A: Ab values in individual serum samples. B: Relationship between anti-β_2-GPI-oxLig-1 Ab values and β_2-GPI-dependent aCL values. C: Relationship between anti-β_2-GPI-oxLig-1 Ab values and anti-β_2-GPI Ab values.

mildly oxidized LDL, cholesteryl hydroperoxyoctadecadienoate (Chol-HPODE) and cholesteryl hydroxyoctadecadienoate (Chol-HODE) were detected as main constituents of oxidation products (52). Chol-HPODE was reported to inactivate platelet-derived growth factor (53). 7-Hydroxycholesterol (both free and esterified) is the major oxysterol formed as an early event in LDL oxidation, with 7-ketocholesterol dominating at later stages (54). Recent studies (55) indicate that elevated plasma levels of 7β-hydroxycholesterol may be associated with an increased risk of atherosclerosis. At later stages in LDL oxidation, cholesteryl or 7-ketocholesteryl esters of 9-oxononanoate derived from cholesteryl linoleate (56) were detected as the most abundant fraction of oxidized cholesteryl linoleate (57, 58). Cholesteryl ester core-aldehydes react with the free ε-amino group of lysines, form complexes with proteins, and were evident to present in human atherosclerotic lesions (58, 59). In the present study, oxLig-1 was identified to be 7-ketocholesteryl-9-carboxynonanoate, one of the oxidation products of cholesteryl linoleate (58) and a major ligand for β_2-GPI. However, it still remains to be elucidated whether or not 7-ketocholesteryl-9-carboxynonanoate is actually present in biological samples as an oxLDL ligand.

Chemically modified LDL can be rapidly taken up by macrophages via receptor-mediated endocytosis, resulting in foam cell formation (44). As models of oxLDL, we used oxLig-1- and PS-liposomes, and studied their binding to J774A.1 cells (Fig. 7). PS-liposomes bind to macrophages via scavenger receptor(s) (42), whereas oxLig-1 did not seem to be a major ligand for scavenger receptors. However, the binding of oxLig-1-liposomes to J774A.1 cells at 4°C increased up to 14 times when oxLig-1-liposomes were added simultaneously with β_2-GPI and WB-CAL-1. The association of oxLig-1-liposomes with J774A.1 cells, at 37°C, also significantly increased upon incubation with β_2-GPI and WB-CAL-1. This binding and uptake might be mediated by the Fcγ receptor. The uptake by macrophages of immune complexes containing oxLDL through the Fcγ

TABLE 1. Binding of anti-β_2-GPI antiphospholipid antibodies (Ab) to lipid-protein complexes in enzyme-linked immunosorbent assay (ELISA)

Exp. 1 Solid-phase lipid (TBARS)[a]	Binding of β_2-GPI-Dependent aCL (Absorbance, mean ± SD, n = 3)				
	Cof-22	EY2C9	WB-CAL-1	APS-1[b]	APS-2[b]
CL (4.46)	1.132 ± 0.025	1.269 ± 0.014	1.361 ± 0.008	2.099 ± 0.216	1.282 ± 0.041
Syn-oxLig-1 (0.66)	1.066 ± 0.114	0.915 ± 0.072	1.035 ± 0.062	1.130 ± 0.177	1.222 ± 0.057
PAPC (107.8)	0.019 ± 0.003	0.011 ± 0.003	0.012 ± 0.001	0.000 ± 0.001	0.010 ± 0.002
oxPAPC[c] (218.3)	0.073 ± 0.007	0.009 ± 0.002	0.015 ± 0.005	0.007 ± 0.002	0.015 ± 0.001
Exp. 2 Added protein	Binding of Protein-Dependent oxLig-1Ab Binding (Absorbance, mean ± SD, n = 3)				
	Cof-22	EY2C9	WB-CAL-1	APS-1[b]	APS-2[b]
w/o	0.050 ± 0.002	0.005 ± 0.001	0.005 ± 0.002	0.012 ± 0.003	0.014 ± 0.001
β_2-GPI	1.007 ± 0.031	1.147 ± 0.028	0.812 ± 0.023	1.043 ± 0.054	0.755 ± 0.024
Nicked β_2-GPI[d]	0.164 ± 0.007	0.005 ± 0.001	0.003 ± 0,001	0.008 ± 0.003	0.011 ± 0.001
Haptoglobin	0.049 ± 0.001	0.006 ± 0.001	0.006 ± 0.001	0.018 ± 0.011	0.015 ± 0.002
Ovalbumin	0.042 ± 0.001	0.004 ± 0.002	0.006 ± 0.002	0.009 ± 0.004	0.016 ± 0.001

In Exp. 1, numbers indicate antibody binding in the presence (15 μg/ml) of β_2-GPI.
In Exp. 2, ELISA was performed using syn-oxLig-1 coated plates in the presence (15 μg/ml) of indicated proteins.
[a] TBARS (thiobarbituric acid reactive substance) is indicated as nmol malondialdehyde equivalent/mg lipid.
[b] APS-1 and APS-2 were β_2-GPI-depleted sera from individual APS patients with episodes of arterial thrombosis.
[c] oxPAPC; PAPC (1-palmitoyl-2-arachidonoyl-phosphatidylcholine) was exposed to air at room temperature for 24 h.
[d] Nicked β_2-GPI was prepared by the plasmin treatment (43).

type 1 receptor transformed macrophages into foam cells (60, 61), and could accelerate the atherogenic process (62–64).

Auto-Abs against a solid phase oxLig-1 complexed with β_2-GPI were detected in sera of APS patients having episodes of arterial thrombosis (Fig. 12). Further, there was a good correlation between values of anti-β_2-GPI-oxLig-1 Abs, anti-β_2-GPI Abs, and β_2-GPI-dependent aCL. In contrast, it was reported that some aPL recognize adducts of oxidized PLs and β_2-GPI (31). In the present study, we have not determined whether oxLig-1 can form covalent adducts with β_2-GPI, and the possibility cannot yet be excluded. However, it is clear that interaction between oxLig-1 and β_2-GPI is essential to express antigenicity for the auto-Abs shown in Table 1, Exp. 2. It was also suggested that domain V, which contains the PL-binding region (15, 16), is distinguished from domains in which epitopes, recognized by aPL in APS patients, locate (38, 65).

In addition to promoting lipid deposition in macrophages, oxLDL has other characteristics that may accelerate atherogenesis. oxLDL is chemotactic for monocytes (66) and for T cells (67), and is cytotoxic for cultured endothelial cells (68). It was also reported that peroxisome proliferator-activated receptor γ (PPARγ), a transcriptional regulator of genes linked to lipid metabolisms, is activated by components of oxLDL, such as 9-HODE, and 13-HODE and that the scavenger receptor, CD36, is upregulated by a combination of PPARγ and retinoid X receptor ligands (69). PPARγ enhances the uptake of oxLDL, thereby promoting foam cell formation. It remains to be determined whether oxLig-1 has any specific influence on nuclear receptors or other biological functions, such as intracellular signal transductions.

In summary, we isolated and characterized a ligand for β_2-GPI present in oxLDL. The ligand (oxLig-1), i.e., 7-ketocholesteryl-9-carboxynonanoate, mediated liposome uptake by macrophages in the presence of β_2-GPI and an anti-β_2-GPI auto-Ab. Our findings on the ligand provide a specific structural and mechanistic link between β_2-GPI and anti-β_2-GPI auto-Abs and atherogenesis in APS.

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