Mapping and Characterization of the Binding Site for Specific Oxidized Phospholipids and Oxidized Low Density Lipoprotein of Scavenger Receptor CD36*

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Recent studies have identified a novel family of oxidized phosphatidylcholines (oxPC<sub>CD36</sub>) that serve as highly specific ligands for scavenger receptor CD36. oxPC<sub>CD36</sub> accumulate in vivo and mediate macrophage foam cell formation as well as promote platelet hyper-reactivity in hyperlipidemia via CD36. The structural basis of oxPC<sub>CD36</sub> binding to CD36 has not been elucidated. We used liquid-phase binding to glutathione S-transferase fusion proteins containing various regions of CD36 to initially identify the region spanning CD36 amino acids 157–171 to contain a major binding site for oxPC<sub>CD36</sub>. A bell-shaped pH profile and salt concentration dependence suggest an electrostatic mechanism of the binding. Two conserved, positively charged amino acids in the region 157–171 (lysines at positions 164 and 166) were identified as critical for oxPC<sub>CD36</sub> and oxidized low density lipoprotein (oxLDL) binding to CD36. Lysine neutralization with chemical modifier or site-directed mutagenesis of lysine 164/166 to alanine or glutamate, but not to arginine, abolished binding. Cells expressing full-length CD36 with mutated lysines (164 and 166) failed to recognize oxPC<sub>CD36</sub> and oxLDL. Synthetic peptides mimicking the CD36 binding site, but not mutated or scrambled peptides, effectively prevented: (i) oxLDL binding to CD36, (ii) macrophage foam cell formation induced by oxLDL, and (iii) platelet activation by oxPC<sub>CD36</sub>. These data indicate that CD36 (160–168) represents the core of the oxPC<sub>CD36</sub> binding site with lysines 164/166 being indispensable for the binding.

CD36 is a 472-amino-acid, 88-kDa heavily glycosylated transmembrane protein that is expressed in various cell types including macrophages, platelets, microvascular endothelial cells, and adipocytes (1, 2). CD36 has been shown to play a significant role in a number of physiological and pathological processes in vivo including atherogenesis, lipid sensing and metabolism, innate immune responses, angiogenesis, uptake of apoptotic cells, and diabetes (2–4). CD36 involvement in such a variety of processes can be partially explained by its capacity to recognize a number of various distinct ligands. Examples of CD36 ligands include thrombospondin-1 (5), oxidized low density lipoproteins (oxLDL) (6), oxidized phospholipids (7–9), fatty acids (10), microbial diacylglycerides (4), hexarelin (3), collagen (11), and malarial parasite-infected erythrocytes (12).

That CD36 can function as a multiligand receptor is conceivable assuming that it has multiple ligand binding domains. Several studies suggest, for example, that the binding sites of thrombospondin-1 and oxLDL on CD36 are different (13, 14). Two distinct binding sites are proposed for oxLDL on CD36. Studies using a monoclonal antibody have shown that domain 155–183 of CD36 plays a critical role in the binding of LDL oxidized by copper (15). Solid phase binding assays using recombinant fusion proteins spanning various regions of CD36, however, implicate the domain 28–93 as the major binding site for oxLDL (16).

We have recently identified a novel family of oxidized choline glycerophospholipids (oxPC<sub>CD36</sub>) that mediate CD36-dependent recognition of LDL oxidized by various pathways. The structural aspect of oxPC<sub>CD36</sub> is essential for high affinity binding to CD36 is an sn-2 acyl group that incorporates a terminal γ-hydroxy(or oxo)-α,β-unaturated carbonyl. A characteristic feature of oxPC<sub>CD36</sub> conformation is a negatively charged distal end of the sn-2 acyl chain residue that partitions into the aqueous phase (17). oxPC<sub>CD36</sub> are formed during the oxidation of LDL by multiple distinct pathways, serve as specific high affinity ligands for CD36 (9), and are present in vivo at sites of enhanced oxidative stress (18–20). OxPC<sub>CD36</sub> mediate foam cell formation induced by oxidized LDL via macrophage CD36 and induce a prothrombotic phenotype in hyperlipidemia via platelet CD36 (18, 20).

In this current study, we investigated the structural basis for the recognition of oxPC<sub>CD36</sub> by CD36 using a combination of site-directed mutagenesis and ligand binding analyses of various GST-CD36 fusion proteins bound to glutathione-Sepharose beads. We demonstrate that amino acids 160–168 of CD36 represent the core of the binding site for oxPC<sub>CD36</sub> and oxidized LDL and that the electrostatic interaction between evolutionary conserved lysines 164 and 166 and oxidized phospholipid moieties is crucial in this binding.

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2 The abbreviations used are: LDL, low density lipoprotein; oxLDL, oxidized LDL; GST, glutathione S-transferase; KdoA-PC and KdoA-PC, 9-keto-10-dodecenoic acid and 5-keto-6-octendioic acid esters of 2-lyso-PC; HA, influenza hemagglutinin epitope tag; HDL, high density lipoprotein; PAPC, 1-hexa-decanoyl-2-ecosa-tetra-5,8,11′, 14′-enoyl-sn-glycero-3-phosphocholine.
EXPERIMENTAL PROCEDURES

General Materials—Tissue culture media and supplements were purchased from Invitrogen. 125I-labeled sodium iodide was purchased from ICN Pharmaceuticals, Inc. (Costa Mesa, CA), and [3H]1,2-dihexadecanoyl-sn-glycerol-3-phosphocholine was from American Radiolabel Chemicals, Inc., (St. Louis, MO). 9-Keto-10-dodecenoic acid (KDiA-PC) and 5-keto-6-oc-tendioic acid esters of 2-lysoPC (KOdiA-PC) were purchased from American Radiolabel Chemicals, Inc. (St. Louis, MO). 9-Keto-10-dodecenoic acid (KDiA-PC) and 5-keto-6-octadecadienioic acid esters of 2-lysoPC (KOdiA-PC) were purchased from Cayman, Inc. (Ann Arbor, MI). CD36 blocking antibody (FA6-152 was obtained from Immunotech-Beckman Coulter. All oligonucleotide primers used for PCR, mutagenesis, or sequencing were made by Integrated DNA Technologies (Coralville, IA). Peptides CD36 long, VQMILNSLINKSKSS (CD36L), CD36 short, SLINKSKSSMF (CD36S), CD36 minimal, SLINKSKSS (CD36m), CD36 164E166E, VQMILNSLINESS (CD36EE), and CD36 scrambled, SILKVNLSQMKLINSI (CD36Scr) were synthesized by Sigma-Genosys (The Woodlands, TX) and purified to >95% as determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. All other reagents were obtained from Sigma unless otherwise specified.

Cloning and Expression of Full-length CD36—For transient expression in mammalian cells, pCGCG-CD36 with a C-terminal HA tag was used. For stable expression in HEK 293T, the CD36 insert with HA tag was cloned into pIRE2-EGFP vector (Clontech).

Cloning, Mutagenesis, Expression, and Purification of GST Fusion Proteins—An array of recombinant GST-CD36 fusion constructs was generated in the bacterial expression vector pGEX3T (16). All the fusion proteins were made in Rosetta(TM)(DE3)pLac strain of Escherichia coli (EMD Biosciences-Novagen, San Diego, CA) and purified by using glutathione-Sepharose 4B beads (GE Healthcare). The size, amount, and purity of the fusion proteins were examined by SDS-PAGE. The molecular weight was found to be close to the predicted value, and purity was typically >95%. PCR-based site-directed mutagenesis of the pGEX3T-CD36 fusion construct or pCGCG-CD36 was carried out by using the QuikChange XL kit (Invitrogen).

Lipoprotein Isolation, Oxidation, and Radiolabeling—LDL was isolated from fresh human plasma by sequential centrifugation, labeled with 125I, and oxidized as described (8). 25 Ci/mg of protein/tube) with radio-labeled ligands in phosphate-buffered saline containing 0.4% bovine serum albumin for 3 h at 25 °C with gentle rocking. Unbound ligands were removed by repeated washing of the beads with phosphate-buffered saline using low speed centrifugation; bound radioactivity was quantified. In competition experiments, unlabeled competitors were added at 20-fold excess. Ligand binding to full-length CD36 was performed using confluent monolayers of cells in 24-well plates. The indicated amounts of 125I-labeled native or modified LDL or high density lipoprotein or [3H]-labeled unilamellar phospholipid vesicles were added in 250 μl of medium containing 1% fetal bovine serum. Following 2 h of incubation at 4 °C, cells were washed three times with medium and lysed by adding 0.1 M NaOH, and then cell-associated radioactivity was quantified. Statistics and Image Analysis—Data represent the mean ± S.D. for the indicated number of samples. Statistical analyses were made using the Student’s t test. Binding parameters for different ligands of CD36 were obtained from non-linear regression analyses in Prism 4 software (GraphPad Software Inc.). Images were captured in TIF format, white points were adjusted, and the final images were sharpened by unsharp masking in PhotoShop CS2.

RESULTS

Mapping of the CD36 Binding Site for oxPC_CD36 with GST-CD36 Deletion Constructs—An array of recombinant GST-CD36 fusion constructs was generated as described (16) and tested in fluid-phase assays for the binding of specific oxidized phospholipids (oxPC_CD36) as well as lipoproteins oxidized by various pathways. The construct that spans CD36 amino acids 118–182 reproducibly showed maximum binding of oxPC_CD36 vesicles and lipoproteins oxidized by various pathways (data for copper-oxidized LDL are shown, Fig. 1A). Two other constructs, GST-CD36_118–182 and GST-CD36_118–182+183, possessing an alternative binding site for oxidized LDL and having significant overlapping regions with GST-CD36_118–182 showed only about 20% of the binding when compared with CD36_118–182, OxPC_CD36 and oxLDL binding to the remaining constructs containing other regions of CD36 was found to be significantly lower (data not shown). This result suggests that the major binding site on CD36 for oxPC_CD36-containing ligands lies between amino acids 157 and 182.
To further characterize GST-CD36\textsubscript{118–182}, and compare it with full-length CD36, we examined its binding properties. oxLDL binding to GST-CD36\textsubscript{118–182} was saturable, having half-maximal binding at 11.2 ± 1.4 μg/ml (≈22 nm), demonstrating high affinity binding (data not shown). Twenty-fold excess unlabeled oxLDL, but not native LDL, significantly inhibited binding, indicating the specificity of the binding (Fig. 1B). Importantly, anti-CD36 antibody FA6-152 (but not iso

type-matched non-immune IgG), which effectively blocks binding of various forms of oxLDL and oxPCCD36 to CD36-overexpressing cells, significantly reduced binding of oxLDL to GST-CD36\textsubscript{118–182} (Fig. 1B). Thrombospondin and collagen type I were reported to be ligands for CD36. However, their binding sites differ from one reported for oxLDL (13, 14, 21, 22). Both failed to compete with oxLDL binding to GST-CD36\textsubscript{118–182}. These results further demonstrated the specificity of the binding and showed that the same binding site for oxPCCD36 operates in full-length CD36 and GST-CD36\textsubscript{118–182}. This was further supported by direct binding experiments. Similar to full-length CD36, GST-CD36\textsubscript{118–182} bound both PAPC vesicles oxidized by myeloperoxidase-generated reactive nitrogen species and vesicles containing synthetic ligand oxPCCD36 (data shown for KDdiA-PC, a prototypic member of oxPCCD36 family (9)) significantly more effectively than vesicles containing native PAPC (Fig. 1C). Under the same conditions, binding of oxPAPC- or KDdiA-PC-containing vesicles by GST-CD36\textsubscript{67–153}, GST-CD36\textsubscript{67–153}, and even GST-CD36\textsubscript{118–182} was significantly lower (Fig. 1C and data not shown). Taken together, these experiments demonstrate that the binding properties of CD36\textsubscript{118–182} mirror those of the full-length cell-expressed CD36.

To further delineate the oxPCCD36 and oxLDL binding domain of CD36, the GST-CD36\textsubscript{118–182} construct was mutated to sequentially delete 9–11 amino acids at a time from the C terminus. These constructs were then used in binding assays performed with ligands containing oxPCCD36 (data for Cu-oxLDL shown, Fig. 1D). The binding activity of the GST fusion protein was unaffected when amino acids 172–182 were deleted but dropped dramatically upon further deletion of amino acids 161–171. This, together with the previous experiment (Fig. 1A), suggests that the binding domain for oxPCCD36 and oxidized LDL on CD36 lies between amino acids 157 and 171.

Nature of the Binding Interaction—To investigate the nature of the ligand binding interaction of oxPCCD36 and CD36, KDdiA-PC binding was performed at varying pH and salt concentrations (Fig. 2A). Under the experimental conditions employed, there was no loss in binding of GST-CD36\textsubscript{118–182} to glutathione-Sepharose beads. Thus, the decrease in bead-associated radioactivity can only be attributed to the actual loss in binding of ligand. A bell-shaped pH profile of the binding (peak at approximately pH 6.5) and a gradual reduction with salt concentration increasing above 0.4 M were observed, indicating involvement of electrostatic interaction.

Aligning various mammalian CD36 protein sequences (Fig. 2B) revealed that the putative binding domain contains two conserved lysine residues at positions 164 and 166. Thus, we hypothesized that these two positively charged residues interact with the negatively charged sn-2 acyl residues of oxPCCD36 and play a critical role in ligand binding. To test this hypothesis, we first used an amine-reactive chemical modifier, N-hydroxysuccinimide. Pretreatment of either GST-CD36\textsubscript{118–182} or CD36-overexpressing HEK 293T cells with N-hydroxysuccinimide resulted in about an 80% reduction in oxLDL binding. This result suggests that lysines are critical in the CD36-mediated oxLDL binding process both in vitro and in vivo.

Mutagenesis of the Putative Binding Site—To further test our hypothesis stated above, we induced systematic mutagenesis of lysines 164 and 166, individually or both, by substitution of the positively charged amino acid to neutral, negative, or positive amino acids, i.e. to alanine, glutamate, or arginine, respectively.
We performed mutagenesis both in GST-CD36_{118–182} as well as in full-length CD36, and binding assays were subsequently carried out using both purified mutated proteins and HEK 293T cells expressing the corresponding full-length mutated CD36. The surface expression of all the mutants in HEK 293T cells was similar as analyzed by surface biotinylation followed by Western blots for HA (data not shown). We observed that substitution of either of the two lysines to negatively charged glutamic acid or neutrally charged alanine effectively reduced binding to the nonspecific level both in GST-CD36_{118–182} and in full-length CD36 (Fig. 2C). At the same time, when the positive charge was retained by a lysine to arginine mutation, binding was affected only slightly (Fig. 2C). Importantly, when other residues in the region 160–168 were mutated individually to alanine, oxLDL binding was not altered (Fig. 2D), strongly suggesting that lysines 164–166 are the only primary requirement for binding.

Peptides Mimicking the CD36 Binding Domain—Four peptides were synthesized to see whether the putative binding domain described above is sufficient for direct interaction to oxLDL (Fig. 2B). Three small overlapping peptides (CD36L, CD36S, and CD36M) that contain the human CD36 binding domain were found to inhibit oxLDL binding to GST-CD36_{118–182} in a dose-dependent fashion, whereas peptides either with substitution of the 164/166 lysines to glutamates (CD36EE) or with a scrambled amino acid sequence (CD36Scr) were far less effective in inhibition (Fig. 3A). The half-maximal inhibition by peptides CD36L, CD36S, and CD36M was achieved at a peptide:GST-CD36 molar ratio of 1 (peptide concentration ~2 μM), implying that they have affinity to oxLDL similar to that of GST-CD36_{118–182} and hence that of full-length CD36. Also, since these three peptides have similar inhibition curves, the amino acid sequence (160–168), which is common to them, seems to be the minimal oxLDL binding domain of CD36.

The CD36 mimicking peptides are a potent inhibitor of oxLDL binding to cells expressing CD36 (Fig. 3B). Interestingly, scrambled peptide (CD36Scr) had no significant effect on the binding while possessing two lysines (Fig. 3B), suggesting that CD36L peptide contains additional required structural features that are lost upon amino acid rearrangement.

High affinity binding of oxidized LDL to CD36 has long been implicated in the formation of lipid-laden foam cells, a critical early cellular event in the atherosclerotic process. In a final set of experiments, we examined whether CD36L can prevent foam cell formation. LDL was oxidized by the physiologically relevant myeloperoxidase/H$_2$O$_2$/NO$_2$ system (8) to generate...
NO₂-LDL, a specific ligand for macrophage scavenger receptor type A (23). NO₂-LDL was then preincubated with CD36L, CD36S, or CD36M peptides and incubated for 24 h with thioglycollate-elicited mouse peritoneal macrophages. Cells were microscopically examined following neutral lipid staining with Oil Red-O and analyzed by bright field microscopy. Representative images of cells are shown. Original magnification, ×400. B, lipid droplets in A were quantified using Image Pro-plus software and expressed as the percentage of the cell area occupied by lipid droplets. C, human platelets isolated by gel filtration were incubated either with KOdiA-PC (5 μM) or with a mixture of KOdiA-PC (5 μM) and peptides (50 μM) for 15 min at room temperature. Platelets were then incubated with fluorescein isothiocyanate-conjugated anti-P-selectin antibody and analyzed by flow cytometry. NA, no addition.

**DISCUSSION**

CD36 is a major contributor to the macrophage uptake of lipoproteins modified by various oxidative pathways and subsequently to foam cell formation *in vitro* and *in vivo* (23, 24). In platelets, CD36 serves as a sensor of specific oxidized phospholipids that accumulate in plasma in hyperlipidemia and mediates platelet hyper-reactivity and a prothrombotic phenotype associated with hyperlipidemia (20). Thus, elucidation and characterization of the binding site on CD36 for oxLDL and specific oxidized phospholipids are important for understanding the molecular mechanisms of cardiovascular disease as well as for the development of preventive therapies.

In this study, we used GST fusion proteins to identify a short linear stretch of amino acids in CD36 that specifically recognizes the novel family of specific oxidized phospholipids oxPCCD36 (9, 18). A characteristic feature of platelets by oxPC<sub>CD36</sub> is an event responsible for platelet hyper-reactivity in dyslipoproteinemias (20). OxPC<sub>CD36</sub> (data for KOdiA-PC shown) induced platelet P-selectin expression and activation of the platelet fibrinogen receptor integrin α<sub>IIb</sub>β<sub>3</sub>, as anticipated. Preincubation of oxPC<sub>CD36</sub> with CD36L or CD36S peptide prevented platelet activation by either ligand (Fig. 4C). Preincubation with CD36Scr peptide did not prevent activation, indicating the specificity of the effect. Importantly, CD36L, CD36S, and CD36Scr had no effect on platelet activation induced by such agonists as ADP and thrombin (Fig. 4C and data not shown). Collectively, these results demonstrate that CD36L and CD36S peptides specifically inhibit the biological activity of oxPC<sub>CD36</sub> and of oxLDL mediated by oxPC<sub>CD36</sub> *in vitro* and further.
Binding Domain of CD36 for oxPC_{CD36}

of oxPC_{CD36} conformation is a negatively charged distal end of the sn-2 acyl chain residue, which was shown recently to be exposed into the aqueous phase (17). We have previously demonstrated that binding of oxPC_{CD36} to CD36 generally increased with increased negative charge of the sn-2 residue (9). In this study, the apparent decrease in binding with increasing salt concentration and a bell-shaped binding curve over a pH range implied that the interaction is primarily electrostatic in nature. The apparent half-maximal binding was observed at pH \( \sim 5.2 \) and 8.9, represented by \( pK_a \) of titratable opposing charge groups. Thus, an electrostatic interaction, presumably between the negatively charged groups in the lipids and the conserved positively charged lysines in CD36, is the primary mechanism of the ligand recognition.

When the lysine 164 and 166 residues of human CD36 were mutated to arginine, binding of oxLDL was unaffected, indicating that the size of the positively charged amino acid is less critical for effective binding. On the other hand, mutagenesis to alanine or glutamic acid completely abolished binding. Taken together these results demonstrate that positive charges at positions 164 and 166 are absolutely critical for CD36 binding ability and that the specific amino acid is less important. Control experiments demonstrated that alanine substitution of amino acids other then Lys-164 and Lys-166 in the region 160–168 resulted in only slight changes in binding property. Previously, similar alanine scanning mutagenesis of another oxLDL receptor, lectin-like oxLDL receptor-1 (LOX-1), showed involvement of basic residues (25). In vitro binding assays using LOX-1 mutants revealed a "basic spine" structure, consisting of linearly aligned arginine residues, that is responsible for ligand binding (26, 27). Since no crystal structure is available for CD36, it is hard to predict further similarities between the oxLDL binding motifs of these two receptors.

Previous studies using a monoclonal antibody directed against the domain 155–183 have shown that this domain of CD36 plays the critical role in the binding of LDL oxidized by copper (15). The same domain has also been implicated in platelet activation, cell adhesion of malaria parasite-infected erythrocytes (1), phagocytosis of apoptotic neutrophils (28), and binding of growth hormone-releasing peptide (29). A later approach has been reported recently where CD36 was blocked by EP 80317, a novel ligand derived from the growth hormone-releasing peptide family (30). EP 80317 induced significant reduction of atherosclerosis in apoE-deficient mice fed Western diet. The disadvantage of such an approach may be inhibition of important physiological functions of CD36, such as delivery of long chain fatty acids to cardiomyocytes and brain (31, 32). Another approach is to specifically inhibit pathological ligands for CD36. Previously, a recombinant, soluble form of CD36 was shown to compete for the binding of oxLDL to membrane-expressed CD36 and to prevent oxLDL-induced adhesion of monocytes to endothelial cells (33). In this study, a short synthetic peptide representing the identified binding domain inhibited biological activity of oxPC_{CD36} and oxidized LDL in macrophage foam cell assays and in a platelet activation assays.

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