Assessment of direct binding interaction between CD36 and its potential lipid ligands using a peptide mimic of the receptor labeled with a fluorophore

Satoshi TSUZUKI¹, Yusaku KIMOTO², Masayuki YAMASAKI³, Tatsuya SUGAWARA⁴, Yuki MANABE⁴, Kazuo INOUE¹, and Tsutomu SASAKI¹

¹Division of Food Science and Biotechnology, Graduate School of Agriculture; ²Department of Food Science and Biotechnology, Faculty of Agriculture, Kyoto University, Oiwake-cho, Kitashirakawa, Sakyoku, Kyoto 606-8502, Japan; ³Department of Food Science and Human Nutrition, Faculty of Agriculture, Ryukoku University, 1–5 Yokotani, Oe-cho, Seta, Otsu, Shiga 520–2194, Japan; and ⁴Division of Applied Biosciences, Graduate School of Agriculture, Kyoto University, Kitashirakawa Oiwake-cho, Sakyoku, Kyoto, 606-8502, Japan

(Received 29 June 2021; and accepted 2 July 2021)

ABSTRACT
Cluster of differentiation 36 (CD36) is a cell-surface receptor that recognizes diverse substances. We have presented indirect evidence that a short segment of the receptor comprising amino acids 149–168 contains a site for binding of its lipid ligands (e.g., distinct fatty acids and aldehydes). However, experimental support for their direct interactions is yet to be achieved. For this, we devised a fluorescence intensity assay, where a synthetic peptide consisting of CD36 amino acids 149–168 labeled with fluorescein isothiocyanate (FITC-CD36¹⁴⁹–¹⁶⁸) and its variant peptides were used as positive and negative probes, respectively. First, we obtained results indicating that 1-palmitoyl-2-(5-keto-6-octenedioyl)phosphatidylcholine (an established CD36 ligand) but not 1-palmitoyl-2-arachidonyl-phosphatidylcholine (a non-ligand of the receptor) bound in a saturable and specific manner to FITC-CD36¹⁴⁹–¹⁶⁸. Strikingly, the assay allowed us to provide the first evidence supporting direct and specific binding between the CD36 segment and fatty aldehydes (e.g., Z-11-hexadecenal). However, this method failed to illustrate specific interactions of the segment with fatty acids, such as oleic acid. Nonetheless, our findings offer further insight into the biologically relevant ligands and the role of CD36. In addition, we suggest that this fluorescence-based technique provides a convenient means to evaluate protein (peptide)-lipid interactions.

INTRODUCTION
Cluster of differentiation 36 (CD36) is a vertebrate protein that is predicted to be membrane-anchored and present on the cell surface via the N- and C-terminal transmembrane domains (PrabhuDas et al. 2017). To date, studies on eutherian mammals such as humans and rodents have illustrated that CD36 consists of 472 amino acid residues, exists in a variety of cell types, recognizes structurally diverse substances, and plays multiple roles (Holmes 2012). For example, CD36 expressed by macrophages/monocytes participates in the capture and clearance of several substances, including oxidized low-density lipoprotein (oxLDL) (Endemann et al. 1993; Park 2014), malaria-infected erythrocytes (Oquendo et al. 1989; Ren 2012), and apoptotic cells (Navazo et al. 1996). Due to its ability to eliminate damaged materials and cells from the blood and whole body, CD36 is classified as a scavenger receptor (recently proposed name: scavenger receptor class B, member 2) (PrabhuDas et al. 2017). CD36 also functions as
a receptor for thrombospondin-1 (TSP-1) and mediates TSP-1-induced apoptosis of endothelial cells to inhibit angiogenesis (Asch et al. 1992; Osz et al. 2014). In addition, CD36 on cells such as adipocytes, cardiac and skeletal myocytes, and small-intestinal enterocytes has long been believed to recognize long-chain fatty acids (LCFAs) to mediate the cellular uptake of the lipid species (Abumrad et al. 1993; Glatz and Luiken 2017). This receptor has recently been found to be even produced by a population of olfactory receptor cells and is abundantly present in the olfactory ciliary layer in mice (Lee et al. 2015b). It has also been postulated to play a role in olfaction in the animals (Lee et al. 2015a, 2017).

OxLDL is a high-affinity ligand for CD36; thus, its binding characteristics to the receptor have been intensively studied (Endemann et al. 1993; Greaves and Gordon 2009, Levitan et al. 2010). Of note are the findings of Podrez and co-workers that (i) distinct forms of glycerophosphatidylcholine in which oxygen atoms are incorporated at the sn-2 acyl chain (or those with a terminal γ-hydroxy(or oxo)-α,β-unsaturated carbonyl at the position) occur on the surface of LDL, upon oxidative processing of the particle and (ii) the oxidized sn-2 acyl chain partitions into the aqueous phase and serves as the essential structural element for the recognition of oxLDL by CD36 (Podrez et al. 2002; Gao et al. 2010); oxidized glycerophosphatidylcholine species capable of being recognized by CD36 are written hereafter as oxGPC\textsuperscript{CD36}. The research group has also shown that (i) the oxLDL/oxGPC\textsuperscript{CD36}-binding site of human CD36 lies within a segment of the extracellular region of the receptor (amino acids 118–182) and (ii) the region spanning amino acids 157–171 contains a major binding site for oxGPC\textsuperscript{CD36} (Kar et al. 2008). In addition to their studies, we have found that (i) a fluorescently labeled oxLDL (FL-oxLDL) binds specifically and saturably to N-terminally biotinylated peptides consisting of residues 149–168 of CD36 immobilized onto a streptavidin-coated solid support (Tsuzuki et al. 2013, 2017a), (ii) oxGPC\textsuperscript{CD36} species, including 1-palmitoyl-2-(5-keto-6-octenediyl)phosphatidylcholine (KODiA-PC), inhibit binding between FL-oxLDL and the peptides (Tsuzuki et al. 2013, 2017a), and (iii) KODiA-PC binds saturably and specifically to amino acids 150–168 of mouse CD36 fused to the C-terminus of glutathione S-transferase (GST) (Tsuzuki et al. 2017b). Our findings suggest that only a short segment of the CD36 molecule is sufficient for the binding of oxLDL/oxGPC\textsuperscript{CD36}.

Using the FL-oxLDL-binding inhibition assay, we have provided evidence that, in addition to oxGPC\textsuperscript{CD36}, a variety of unsaturated LCFAs, including oleic and linoleic acids, and distinct straight-chain, aliphatic aldehydes, such as Z-11 hexadecenal and tridecanal, can interact with amino acids 150–168 (or 149–168) of CD36 (Kozai et al. 2014; Takai et al. 2014; Tsuzuki et al. 2016a, 2016b, 2017a, 2018). However, we were unable to rule out the possibility that these fatty acids and aldehydes merely cause denaturation and/or degradation of FL-oxLDL, resulting in the failure of the binding of the particles to CD36 peptides. In other words, it remains uncertain whether these aliphatic lipids indeed bind to the short segment of CD36.

The aim of this study was to provide experimental support for direct binding interactions between CD36 and its potential lipid ligands. For this purpose, we adopted a fluorescence-based method, which is often referred to as “fluorescence intensity assay” (Dadgar et al. 2013). This assay method utilizes the emission properties of fluorophores — an increase in emission intensity with an increase in hydrophobicity in the immediate environment (Loving et al. 2009) and has thus been applied for assessment of protein-lipid interactions. For example, increased fluorescence emission has been detected upon exposure of a fluorescent-labeled peroxisome proliferator-activated receptor agonist (a labeled lipid ligand) to the hydrophobic binding pocket of the respective receptor (Ban et al. 2012).

In this study, we labeled the receptor side (a short synthetic peptide) with a fluorophore and used it as a probe (Fig. 1). Even under such conditions, increased fluorescence readings are expected to be recorded upon binding of a ligand (a lipid molecule) to the receptor (the labeled probe) (Fig. 1A). In other words, binding of a lipid molecule to the peptide moiety of the probe would bring an increase in the hydrophobicity of the environment surrounding the dye moiety. A significant advantage of this method compared to previous oxLDL-binding inhibition assays is that no additional components other than receptor and ligand are included in the assay mixture, thus allowing a direct and coherent assessment of their interactions. In this study, by means of the assay, we provide the first evidence for a direct binding interaction between amino acids 150–168 of CD36 and aliphatic aldehydes such as Z-11-hexadecenal. This study also challenged the binding of LCFAs to the CD36 site.
MATERIALS AND METHODS

Test lipids. The purity of the test lipids used was >95%. KOdiA-PC and 1-palmitoyl-2-arachidonylphosphatidylcholine (PAPC) were purchased from Cayman Chemical (Ann Arbor, MI, USA) (note that KOdiA-PC is supplied in ethanol solution at 7.5 mM). Benzaldehyde, Z-11-hexadecenal, linoleic acid, oleic acid, palmitic acid, stearic acid, and tri-decanal were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Octanal and vanillin were purchased from Tokyo Chemical Industry (Tokyo, Japan). The test lipids other than KOdiA-PC were dissolved in ethanol at a concentration of 40 mM (for those other than PAPC) or 7.5 mM (for PAPC) and stored at –20°C until use.

Preparation of the mimic peptides for CD36. An oligopeptide containing amino acid residues 149–168 of mouse CD36 with fluorescein isothiocyanate (FITC) at the N-terminus (designated as FITC-CD36_{149–168}, Fig. 1B) and its respective variant peptides (Scr-FITC-CD36_{149–168} and 3K/NDE-FITC-CD36_{149–168}, Fig. 1B) were synthesized by and purchased from Life Technologies Japan (Tokyo). The Scr-FITC-CD36_{149–168} peptide was used as a negative control. The 3K/NDE-FITC-CD36_{149–168} peptide, in which Lys residues at positions corresponding to 163, 164, and 166 of CD36 were substituted with Asn, Asp, and Glu residues, respectively, was used as another negative control. Note that the positively charged residues at positions 163, 164, and 166 are indispensable for the interaction of the receptor with oxLDL (Kar et al. 2008; Takai et al. 2013, 2014).

Five additional Ser residues were added to each of the FITC-conjugated peptides on the N-terminal side in the expectation that the addition of a few successive Ser residues would improve the solubility in aqueous solutions. The FITC-conjugated peptides were dissolved in dimethyl sulfoxide at a concentration of 1 mM and stored at –20°C until use.

Fluorescence intensity assay. The solutions of the test lipids in ethanol were sequentially diluted with ethanol. A 5 μL aliquot of the diluted lipids was placed in a 1.5 mL microcentrifuge tube, to which was added 95 μL of a concentrated solution of phosphate buffered saline (PBS, 8 mM Na₂HPO₄, 1.5 mM
RESULTS

Utility assessment of the fluorescence intensity assay using KOdiA-PC as an established ligand of CD36

Using a polyacrylamide gel electrophoresis-based assay, we have shown that (i) KOdiA-PC (Fig. 2A), but not its non-oxidized counterpart, PAPC (Fig. 2A), binds in a saturable, concentration-dependent manner to a GST-fusion protein that includes amino acids 150–168 of mouse CD36 at the C-terminus (GST-CD36150–168) and that (ii) the oxGPCCD36 (and PAPC) does not bind to GST alone or a variant of GST-CD36150–168 in which Lys residues at positions 164 and 166 are mutated to Glu residues (Tsuzuki et al. 2017b). The previous findings have further validated that KOdiA-PC but not PAPC binds directly and specifically to the short segment of CD36.

Using KOdiA-PC and PAPC as a ligand and non-ligand of CD36, respectively, we assessed the utility of our fluorescence intensity assay (Fig. 1A). Each of the labeled peptides was included in the assay mixture at a concentration of 10 nM to provide the conditions under which the concentrations of test lipids greatly exceeded those of the peptides. The peptides were incubated for a period with KOdiA-PC over a concentration range of 2.9 to 375 μM. The concentration range was set in reference to that the EC50 for binding of KOdiA-PC to GST-CD36150–168 was tens of micromolar concentration (Tsuzuki et al. 2017b). For comparison, we evaluated the behavior of PAPC toward the peptides using the same concentration range as that for KOdiA-PC.

Plots of FITC-CD36149–168 fluorescence as a function of KOdiA-PC concentration exhibited a saturating sigmoidal profile (Fig. 2B, upper panel). Curve fitting to the data revealed an EC50 of 18 μM (Hill slope, 1.3) (Table 1). Our interpretation is that the oxGPCCD36 species bound saturably to the peptide. On the other hand, fluorescence readings for Scr- and 3K/NDE-FITC-CD36149–168 peptides increased hyperbolically with increasing KOdiA-PC concentrations up to 47 μM and then decreased gradually (Fig. 2B, upper panel). A hypothesis for the bell-shaped pattern of plots with variant peptides is described in the Discussion section. In any event, these results suggest that the interaction of KOdiA-PC with FITC-CD36149–168 is specific, which depends essentially on the amino acid sequence and positively charged residues of the peptide.

The plots of FITC-CD36149–168 fluorescence versus PAPC concentration did not yield a saturating pattern (Fig. 2B, lower panel). From an exponential curve, the EC50 value was calculated to be more than 10 mM (Table 1). Curves fitting to the data for the variant peptides were also exponential (Fig. 2B, lower panel), suggesting that PAPC binds non-specifically and very weakly to FITC-CD36149–168.

Taken together, we interpret these results to indicate that KOdiA-PC but not PAPC binds in a saturable and specific manner to FITC-CD36149–168. We also propose that our fluorescence intensity assay using the FITC-conjugated peptides as probes can be applied as a method to assess direct binding interactions between amino acids 149–168 of CD36 and potential lipid ligands of the receptor.

Assessment of binding interaction of FITC-labeled peptides with molecules having an aldehyde moiety

Our oxLDL-binding inhibition assay has allowed the identification of several straight-chain aliphatic aldehydes as potential lipid ligands of CD36 (Tsuzuki et al. 2016a, 2016b, 2017a, 2018). The aldehydes identified include Z-11-hexadecenal (sixteen-carbon aldehyde) (Fig. 3A), which functions as a male-attracting pheromone in distinct species of insects (Hedin et al. 1986). Drosophila is known to perceive the aldehyde via a mechanism that requires a sensory neuron membrane protein named SNMP-1, an insect homolog of CD36 (Benton et al. 2007). More notably,
Direct CD36-lipid interactions

Z-11-hexadecenal was demonstrated to bind to the ectodomain of CD36 via a surface plasmon resonance technique (Gomez-Diaz et al. 2016). However, no information is available on the binding site for the fatty aldehyde. In addition, control experiments using structurally distinct proteins or the mutants of CD36 were absent. Therefore, the binding specificity remains to be ascertained. Our assay can overcome these limitations.

Z-11-hexadecenal over a concentration range of 0.49 to 300 μM increased the fluorescence emission of FITC-CD36_{149-168} in a saturable manner, with EC_{50} and Hill slope of 7.6 μM and 1.1, respectively (Fig. 3B, left panel and Table 1). The plots for Scr-FITC-CD36_{149-168} and 3K/NDE-FITC-CD36_{149-168} displayed an exponential pattern (Fig. 3B, left panel).
These results suggest that Z-11-hexadecenal binds to FITC-CD36<sub>149–168</sub>, relying on the amino acid sequence and positively charged residues of the peptide and provide the first indication that the short region of CD36 serves as one of the sites for recognition of the alkenal.

Saturated aliphatic aldehydes with 9–16 carbon atoms, any of which occur in certain species of plants as odor-active volatiles (their biological roles remain ambiguous), have also been postulated to be candidate ligands of CD36 (Tsuzuki et al. 2017a), although no studies have challenged the direct binding interaction between the receptor and aldehydes. We investigated the reactivity to the FITC-labeled peptides of tridecanal (thirteen-carbon alkanal), which displayed the highest activity among saturated fatty aldehydes tested in the oxLDL-binding inhibition assay (Tsuzuki et al. 2017a). The results were essentially the same as those of Z-11-hexadecenal (Fig. 3B, right panel), suggesting a direct and specific binding between FITC-CD36<sub>149–168</sub> and the alkanal. The EC<sub>50</sub> for tridecanal differs substantially from that for Z-11-hexadecenal (Table 1), indicating that the structure of hydrocarbon chains affects the ability of fatty aldehydes to interact with amino acids 149–168 of CD36. Plots of FITC-CD36<sub>149–168</sub> fluorescence versus the concentration of octanal (eight-carbon alkanal), which displayed no detectable activity in the oxLDL-binding inhibition assay (Tsuzuki et al. 2017a), exhibited an exponential profile (Supplementary Fig. S1 and Table 1).

We have found that aromatic aldehydes (or benzenoids with an aldehyde group), like benzaldehyde and vanillin (Fig. 3A), which are found in certain species of plants and are often used as synthetic products in the industries for cosmetic purposes or as flavoring additives, show little or no ability to inhibit FL-oxLDL binding to peptide mimics of CD36 (Tsuzuki et al. 2017a). We investigated the effects of benzaldehyde and vanillin on FITC-CD36<sub>149–168</sub> fluorescence over a broad concentration range (0.26 to 2,000 µM), but no characteristic sigmoid plots were given in either case (Fig. 3C). Each of the EC<sub>50</sub> values for aromatic aldehydes was estimated to be large (Table 1). These aldehydes might, if any, bind inefficiently to the peptide, which is consistent with the data from the oxLDL-binding inhibition assay. The results obtained with aromatic aldehydes reinforce the significance of aliphatic hydrocarbon chains as structural elements for recognition by CD36.

Assessment of binding interaction of amino acids 149–168 of CD36 with LCFAs
CD36 is believed to recognize LCFAs and facilitate their cellular entry (Abumrad et al. 1993; Glatz and

### Table 1 Assessment and comparison of the abilities of phospholipids, fatty aldehydes, aromatic aldehydes, unsaturated LCFAs, and saturated LCFAs to interact with FITC-conjugated peptides

| Lipids                  | FITC-CD36<sub>149–168</sub> | Scr-FITC-CD36<sub>149–168</sub> | 3K/NDE-FITC-CD36<sub>149–168</sub> |
|-------------------------|-----------------------------|---------------------------------|-----------------------------------|
|                         | EC<sub>50</sub> (µM) | Hill slope | R<sup>2</sup> | EC<sub>50</sub> (µM) | Hill slope | R<sup>2</sup> | EC<sub>50</sub> (µM) | Hill slope | R<sup>2</sup> |
| (Phospholipids)         |                            |                                |                                    |
| KDiA-PC                 | 18                          | 1.3                             | 0.93                               |
|                          |                             | 5.9                             | 2.0                               | 0.86       | 5.1             | 3.0             | 0.83       |
| PAPC                    | > 10,000                    | 0.55                            | 0.97                               |
|                          |                             | > 10,000                        | 0.49                               | 0.96       | > 10,000        | 0.58            | 0.97       |
| (Fatty aldehydes)       |                            |                                |                                    |
| Z-11-Hexadecenal        | 7.6                         | 1.1                             | 0.97                               |
|                          |                             | > 10,000                        | 0.42                               | 0.98       | > 10,000        | 0.52            | 0.94       |
| Tridecanal              | 35                          | 2.4                             | 0.97                               |
|                          |                             | > 10,000                        | 0.57                               | 0.96       | 190             | 0.79            | 0.92       |
| Octanal                 | 9900                        | 0.28                            | 0.89                               |
| (Aromatic aldehydes)    |                            |                                |                                    |
| Benzaldehyde            | > 10,000                    | 0.043                           | 0.69                               |
|                          |                             | N.T.                            |                                    |
| Vanillin                | (> 10,000)                  | (160)                           | (0.0)                              |
|                          |                             | N.T.                            |                                    |
| (Unsaturated LCFAs)     |                            |                                |                                    |
| Oleic acid              | 20                          | 3.6                             | 0.96                               |
|                          |                             | 18                              | 3.6                               | 0.96       | 21              | 3.7             | 0.93       |
| Linoleic acid           | 21                          | 2.1                             | 0.87                               |
|                          |                             | 18                              | 1.6                               | 0.90       | 19              | 2.9             | 0.77       |
| (Saturated LCFAs)       |                            |                                |                                    |
| Palmitic acid           | (8.7)                       | (76)                            | (0.34)                             |
|                          |                             | N.T.                            |                                    |
| Stearic acid            | (20)                        | (34)                            | (0.10)                             |
|                          |                             | N.T.                            |                                    |

Values calculated from the progress curves that poorly fit the theoretical ones (i.e., when the determination coefficient, R<sup>2</sup>, is less than 0.50) are presented in parenthesis for reference. N.T.; not tested.
Direct CD36-lipid interactions

Fig. 3 Assessment of binding interaction of amino acids 149–168 of CD36 with fatty and aromatic aldehydes by fluorescence intensity assay. A Structure of Z-11-hexadecenal, tridecanal, benzaldehyde, and vanillin. B Profiles of fluorescence readings for FITC-CD36<sub>149–168</sub>, Scr-FITC-CD36<sub>149–168</sub> (Scr variant), and 3K/NDE-FITC-CD36<sub>149–168</sub> (3K/NDE variant) in the incubation with Z-11-hexadecenal or tridecanal. C Profile of fluorescence readings for FITC-CD36<sub>149–168</sub> in the incubation with benzaldehyde or vanillin. These aldehydes were included in assay mixture within the concentration range indicated. After 1 h incubation of the mixtures, the fluorescence was measured as described in MATERIALS AND METHODS. In each of the plots, the mean fluorescence of wells exposed to assay mixture containing the aldehyde at the minimum concentration was set at 1.0. The lines for benzaldehyde and vanillin were illustrated by a dotted one. Data are expressed as mean ± S.D. for four independent wells.

Luiken 2017). To date, however, only a few studies have addressed the direct binding between the receptor and LCFAs. For instance, in an earlier study, Baillie et al. (1996) challenged the binding interaction by a method whereby unbound LCFAs were removed with a lipid-capturing resin from a mixture consisting of LCFAs and CD36 isolated from adipocytes. More recently, surface plasmon resonance analysis has enabled characterization of the binding between a recombinant extracellular domain of CD36 and LCFAs (Jay et al. 2015). In either case, however, information about the binding site(s) to the
CD36 149–168 (Table 1). Linoleic acid (or $Z,Z$-9,12-octadecadienoic acid, C18:2), another unsaturated LCFA species, gave essentially the same results as oleic acid in that EC50 values for the three peptides were comparable (Fig. 4A, right panel, and Table 1). Altogether, we suggest that FITC-CD36 149–168 directly binds these LCFAs but that the binding is non-specific and does not rely exclusively on the amino acid sequence and positively charged residues of the peptide. Alternatively, this method per se may not be suited to address specific binding interactions between the amino acid 149–168 of CD36 and the lipids.

Saturated LCFAs, palmitic acid (or hexadecanoic acid, C16:0) and stearic acid (or octadecanoic acid, C18:0), had little influence on the fluorescence emission of FITC-CD36149–168 when applied in the same concentration range as that used for oleic and linoleic acids (Fig. 4B and Table 1). This implies that the LCFAs have little interaction with the receptor for LCFAs is still unavailable.

Among unsaturated LCFAs capable of inhibiting FL-oxLDL binding to a CD36 peptide, oleic acid (or $Z$-9-octadecenoic acid, C18:1) displayed the highest activity (Takai et al. 2014). We analyzed the behavior of the LCFA toward the FITC-conjugated peptides. To avoid severe detergent effects of LCFAs, oleic acid was added to the assay mixture at concentrations of less than 100 μM (the concentration range of 5.9 to 100 μM). In an assay with FITC-CD36149–168, the relationship between the fluorescence measured and the concentration of oleic acid was sigmoidal (Fig. 4A, left panel), suggesting a saturable binding of the lipid to the peptide. However, in contrast to the results from KOdiA-PC, Z-11-hexadecenal, and tridecanal, the plots for the Scr- and 3K/NDE-FITC-CD36149–168 peptides exhibited a sigmoidal pattern (Fig. 4A, left panel). Particularly emphasized are the similar values of EC50 and Hill slope for the variant peptides to those for FITC-CD36149–168 (Table 1). Linoleic acid (or $Z,Z$-9,12-octadecadienoic acid, C18:2), another unsaturated LCFA species, gave essentially the same results as oleic acid in that EC50 values for the three peptides were comparable (Fig. 4A, right panel, and Table 1). Altogether, we suggest that FITC-CD36149–168 directly binds these LCFAs but that the binding is non-specific and does not rely exclusively on the amino acid sequence and positively charged residues of the peptide. Alternatively, this method per se may not be suited to address specific binding interactions between the amino acid 149–168 of CD36 and the lipids.

Saturated LCFAs, palmitic acid (or hexadecanoic acid, C16:0) and stearic acid (or octadecanoic acid, C18:0), had little influence on the fluorescence emission of FITC-CD36149–168 when applied in the same concentration range as that used for oleic and linoleic acids (Fig. 4B and Table 1). This implies that the LCFAs have little interaction with the pep-
tide. Further, this coincides with their inability to inhibit FL-oxLDL binding to peptide mimics of CD36 (Kozai et al. 2014; Takai et al. 2014; Tsuzuki et al. 2017a).

**DISCUSSION**

In this study, we found that our fluorescence intensity assay, in which a protein (peptide) is labeled with a fluorophore, could be used to assess protein-lipid interactions. This is exemplified by the fact that KOdiA-PC, an established lipid ligand of CD36, increased the fluorescence emission of FITC-CD36\textsubscript{149-168} in a concentration-dependent, saturable manner (Fig. 2B). The oxidized phospholipid may be able to come close enough to the dye moiety of FITC-CD36\textsubscript{149-168} upon its binding to the peptide moiety of the probe, resulting in increased hydrophobicity around the dye (Fig. 1A). This seems to have been possible because the peptide used was short. It is noted that in this system, parallel assays using variant peptides allow to concurrently evaluate the specificity of interaction. We believe that our application is useful to readily and promptly evaluate specific binding interactions between short peptide fragments and lipids. In addition, this assay system would offer an effective platform for screening of target lipids not only of CD36 but also of distinct proteins that are capable of ligand recognition with only a short segment.

It remains unclear why the plots of fluorescence value versus KOdiA-PC concentration displayed a bell-shaped profile in the titration of Scr- and 3K/NDE-FITC-CD36\textsubscript{149-168} peptides. We previously obtained evidence that KOdiA-PC formed assemblies (or possibly micelles) consisting of a few to several molecules in isotonic solutions and predicted that the oxGPC\textsubscript{CD36} species in such states complexed with GST-CD36\textsubscript{150-168} (Tsuzuki et al. 2017b). By analogy, it is possible that the FITC-CD36\textsubscript{149-168} peptide can only recognize the oxidized phospholipid in the assembled states. On the other hand, the variant peptides of FITC-CD36\textsubscript{149-168} are assumed to be incapable of binding KOdiA-PC assemblies, based on our previous observations that neither GST alone nor a point mutated variant of GST-CD36\textsubscript{150-168} formed a complex with KOdiA-PC (i.e., its assemblies) (Tsuzuki et al. 2017b). Overall, we speculate that KOdiA-PC exists mainly as a single molecule when included at lower concentrations in isotonic solution, and the lipids in such a state gain access to Scr- and 3K/NDE-FITC-CD36\textsubscript{149-168} (but probably not to FITC-CD36\textsubscript{149-168}) to intensify the fluorescence emission. However, the number of solitary KOdiA-PC molecules decreases upon formation of the assemblies (or incorporation into them) with increasing concentration of the lipid, accounting for the concentration-dependent decreases in fluorescence readings. Further studies are required to confirm this hypothesis.

A clear indication was given for a direct and specific binding interaction between FITC-CD36\textsubscript{149-168} and Z-11 hexadecenal (Fig. 3B). By comparing the data from the fatty aldehyde with those from unsaturated LCFAs, the terminal aldehyde functional group is considered a structural element governing the specificity to FITC-CD36\textsubscript{149-168}. However, we cannot exclude the possibility that both aldehyde and hydrocarbon chain moieties may be indispensable for determining the specificity. Note that the EC\textsubscript{50} value for the increase of FITC-CD36\textsubscript{149-168} fluorescence by Z-11-hexadecenal was lower than that by KOdiA-PC (Table 1), implying a higher affinity for the peptide than the oxGPC\textsubscript{CD36}. We need to investigate the underlying mechanisms (i.e., amino acid residues) required for the binding of these structurally distinct lipid molecules.

Our fluorescence-based assay did not permit us to define a specific binding interaction between amino acids 149–168 of CD36 and unsaturated LCFAs. Indeed, the lipids tested behaved toward the three FITC-conjugated peptides in a comparable manner (Fig. 4A). The most unexpected finding was that the lipid species did not appear to rely on charged residues in peptides for interaction. One hypothesis is that unsaturated LCFAs (possibly as monomers) bind to FITC-CD36\textsubscript{149-168} via hydrophobic interactions between the hydrocarbon chains of the lipids and the hydrophobic amino acid side chains of the probes, and that the binding does not depend exclusively on the side-by-side arrangement of hydrophobic residues (Fig. 1B). This idea is supported by the fact that the monomers of unsaturated LCFAs bind to and form a complex with structurally diverse proteins (e.g., α-lactalbumin, β-lactoglobulin, and lysozyme) via mechanisms involving hydrophobic interaction (Brinkmann et al. 2013). However, this assumption does not account well for the specificity to FITC-CD36\textsubscript{149-168} of Z-11-hexadecenal, which has a hydrocarbon chain moiety similar to that of oleic acid. It is possible that, unlike the fatty aldehyde, the unsaturated LCFAs form micelles and incorporate the positive and negative probes to similar degrees under the conditions employed. It should be determined in the future whether the observed phenomena are due to the fatty acid monomers or mi-
cells. In addition, future studies are needed to address whether the binding between the amino acids 149–168 of CD36 and the monomers of unsaturated LCFAs, if any, has some biological significance, such as contributing to the cellular uptake of the lipids.

Data were collected on binding to CD36 of palmitic acid (Jay et al. 2015) and stearic acid (Baillie et al. 1996). On the other hand, we found no evidence that these saturated LCFAs bind to amino acid 149–168 of CD36. One explanation for this discrepancy is that this receptor binds saturated LCFAs at sites other than the short segment. Multiple sites on the receptor have been predicted to recognize LCFAs (Tarhda et al. 2013). In addition, previous studies on the binding between CD36 and LCFAs have been conducted using assay mixtures containing a non-ionic detergent (Baillie et al. 1996) or a form of cyclodextrin (Jay et al. 2015) for solubilization and dispersion of lipids. It is therefore possible that under our experimental conditions where no such agents are included, these saturated LCFAs could only disperse as amorphous aggregates in the assay mixture and were therefore unable to interact with FITC-CD36 149–168. Our assay system needs to be improved in this context.

In summary, by an assay using a fluorescent-labeled peptide mimic of CD36, we provided the first evidence for a direct and specific binding interaction of a short segment of the receptor (amino acids 149–168) with some of the aliphatic aldehydes. On the other hand, specific binding interactions between the short segment of the receptor and LCFAs remain to be addressed. Nonetheless, the present findings would expand our knowledge regarding the ligand specificity of CD36. A study is underway to search for additional potential CD36 ligands by means of our fluorescence intensity assay, and it will provide further insights into the role of CD36.

Acknowledgments

We would like to thank Editage (www.editage.jp) for the English language review. This work was supported by JSPS KAKENHI Grant (C) Number 16K07733 and 19K05912 to S.T.

REFERENCES

Abumrad NA, el-Maghrabi MR, Amri EZ, Lopez E and Grimaldi PA (1993) Cloning of a rat adipocyte membrane protein implicated in binding or transport of long-chain fatty acids that is induced during preadipocyte differentiation. Homology with human CD36. J Biol Chem 268, 17665–17668.

Asch AS, Silbiger S, Heimer E and Nachman RL (1992) Thrombospondin sequence motif (CSVTSC) is responsible for CD36 binding. Biochim Biophys Res Commun 182, 1208–1217.

Baillie AGS, Coburn CT and Abumrad NA (1996) Reversible binding of long-chain fatty acids to purified FAT, the adipose CD36 homolog. J Membr Biol 153, 75–81.

Ban S, Oyama T, Kasuga J, Ohgane K, Nishio Y, et al. (2012) Bidirectional fluorescence properties of pyrene-based peroxisome proliferator-activated receptor (PPAR) α/δ dual agonist. Biosorg Med Chem 20, 3460–3464.

Benton R, Vannice KS and Voshall LB (2007) An essential role for a CD36-related receptor in pheromone detection in Dro sophila. Nature 450, 289–293.

Brinkmann CR, Thiel S and Otzen DE (2013) Protein-fatty acid complexes: biochemistry, biophysics and function. FEBs J 280, 1733–1749.

Dadgar S, Ramjan Z and Floriano WB (2013) Paclitaxel is an inhibitor and its boron dipyrromethene derivative is a fluorescent recognition agent for Botulimum neurotoxin subtype A. J Med Chem 56, 2791–2803.

Endemann G, Stanton LW, Maddern KS, Bryant CM, White RT, et al. (1993) CD36 is a receptor for oxidized low density lipoprotein. J Biol Chem 268, 11811–11816.

Gao D, Ashraf MZ, Kar NS, Lin D, Sayre LM, et al. (2010) Structural basis for the recognition of oxidized phospholipids in oxidized low density lipoproteins by class B scavenger receptors CD36 and SR-BI. J Biol Chem 285, 4447–4454.

Glatz JFC and Luiken JJFP (2017) From fat to FAT (CD36/SREBP-2): Understanding the regulation of cellular fatty acid uptake. Biochimie 136, 21–26.

Gomez-Diaz C, Bargeton B, Abuin L, Bukar N, Reina JH, et al. (2016) CD36 ectodomain mediates insect pheromone detection via a putative tunneling mechanism. Nat Commun 15, 11866.

Greaves DR and Gordon S (2009) The macrophage scavenger receptor at 30 years of age: current knowledge and future challenges. J Lipid Res 50 (Suppl), S282–S286.

Hedin PA, Davis FM, Dickens JC, Burks ML, Bird TG, et al. (1986) Identification of the sex attractant pheromone of the southwestern corn borer Diatraea grandiosella Dyar. J Chem Ecol 12, 2051–2063.

Holmes RS (2012) Comparative studies of vertebrate platelet glycoprotein 4 (CD36). Biomolecules 2, 389–414.

Jay AG, Chen AN, Paz MA, Hung JP and Hamilton JA (2015) CD36 binds oxidized low density lipoprotein (LDL) in a mechanism dependent upon fatty acid binding. J Biol Chem 290, 4590–4603.

Kar NS, Ashraf MZ, Valiyaveettil M and Podrez EA (2008) Mapping and characterization of the binding site for specific oxidized phospholipids and oxidized low density lipoprotein of scavenger receptor CD36. J Biol Chem 283, 8765–8771.

Kozai Y, Tsuzuki S, Takai M, Eguchi A, Matsumura S, et al. (2014) Further validation of unsaturated long-chain fatty acids as inhibitors for oxidized low-density lipoprotein binding to CD36 via assays with synthetic CD36 peptide-cross-linked plates. Biosci Biotechnol Biochem 78, 839–842.

Lee S, Eguchi A, Sakamoto K, Matsumura S, Tsuzuki S, et al. (2015a) A role of CD36 in the perception of an oxidized phospholipid species in mice. Biomed Res (Tokyo) 36, 303–311.

Lee S, Eguchi A, Tsuzuki S, Matsumura S, Inoue K, et al. (2015b) Expression of CD36 by olfactory receptor cells and its abundance on the epithelial surface in mice. PLoS One
Direct CD36-lipid interactions

10, e0133412.
Lee S, Tsuzuki S, Amitsuka T, Masuda D, Yamashita S, et al. (2017) CD36 involvement in the olfactory perception of oleic aldehyde, an odour-active volatile compound, in mice. *Biomed Res (Tokyo)* 38, 207–213.

Levitan I, Volkov S and Subbaiah PV (2010) Oxidized LDL: diversity, patterns of recognition, and pathophysiology. *Antiox Red Signal* 13, 39–75.

Loving GS, Sainlos M and Imperiali B (2009) Monitoring protein interactions and dynamics with solvatochromic fluorophores. *Trends Biotechnol* 28, 73–83.

Navazo MD, Daviet L, Savill J, Ren Y, Leun LL, et al. (1996) Identification of a domain (155–183) on CD36 implicated in the phagocytosis of apoptotic neutrophils. *J Biol Chem* 271, 15381–15385.

Oquendo P, Hundt E, Lawler J and Seed B (1989) CD36 directly mediates cytoadherence of Plasmodium falciparum parasitized erythrocytes. *Cell* 58, 95–101.

Oszt K, Ross M and Petrik J (2014) The thrombospondin-1 receptor CD36 is an important mediator of ovarian angiogenesis and folliculogenesis. *Reprod Biol Endocrinol* 12, 21.

Park YM (2014) CD36, a scavenger receptor implicated in atherosclerosis. *Exp Mol Med* 46, e99.

Podrez EA, Poliakov E, Shen Z, Zhang R, Deng Y, et al. (2002) Identification of a novel family of oxidized phospholipids that serve as ligands for the macrophage scavenger receptor CD36. *J Biol Chem* 277, 38503–38516.

PrabhuDas MR, Baldwin CL, Bollyky PL, Bowdish DME, Drickamer, et al. (2017) A consensus definitive classification of scavenger receptors and their roles in health and disease. *J Immunol* 198, 3775–3789.

Ren Y (2012) Peroxisome proliferator-activator receptor γ: A link between macrophage CD36 and inflammation in malaria infection. *PIAR Res* 2012, 640769.

Takai M, Tsuzuki S, Matsuno Y, Kozai Y, Eguchi A, et al. (2013) Assessment of key amino-acid residues of CD36 in specific binding interaction with an oxidized low-density lipoprotein. *Biosci Biotechnol Biochem* 77, 1134–1137.

Takai M, Kozai Y, Tsuzuki S, Matsuno Y, Fujikawa M, et al. (2014) Unsaturated long-chain fatty acids inhibit the binding of oxidized low-density lipoproteins to a model CD36. *Biosci Biotechnol Biochem* 78, 238–244.

Tarhda Z, Semlali O, Kettani A, Moussa A, Abumrad NA, et al. (2013) Three dimensional structure prediction of fatty acid binding site on human transmembrane receptor CD36. *Bioinform Biol Insights* 7, 369–373.

Tsuzuki S, Takai M, Matsuo Y, Kozai Y, Fujikawa M, et al. (2013) A synthetic peptide-based assay system for detecting binding between CD36 and an oxidized low-density lipoprotein. *Biosci Biotechnol Biochem* 77, 132–137.

Tsuzuki S, Amitsuka T, Okahashi T, Kozai Y, Matsumura S, et al. (2016a) A single aldehyde group can serve as a structural element for recognition by transmembrane protein CD36. *Biosci Biotechnol Biochem* 80, 1375–1378.

Tsuzuki S, Amitsuka T, Okahashi T, Kozai Y, Yamasaki M, et al. (2016b) Identification of the odor-active volatile compound (Z,Z)-4,7-tridecadienal as a potential ligand for the transmembrane receptor CD36. *Biosci Res (Tokyo)* 37, 335–342.

Tsuzuki S, Amitsuka T, Okahashi T, Kimoto Y and Inoue K (2017a) A search for CD36 ligands from flavor volatiles in foods with an aldehyde moiety: identification of saturated aliphatic aldehydes with 9–16 carbon atoms as potential ligands of the receptor. *J Agric Food Chem* 65, 6647–6655.

Tsuzuki S, Yamasaki M, Kozai Y, Sugawara T, Manabe Y, et al. (2017b) Assessment of direct interaction between CD36 and an oxidized glycerophospholipid species. *J Biochem* 162, 163–172.

Tsuzuki S, Kimoto Y, Lee S, Sugawara T, Manabe Y, et al. (2018) A novel role for scavenger receptor B1 as a contributor to the capture of specific volatile odorants in the nasal cavity. *Biomed Res (Tokyo)* 39, 117–129.
Supplementary Fig. S1  Assessment of binding interaction between CD36 amino acids 149–168 and a straight-chain, aliphatic aldehyde, octanal, by fluorescence intensity assay. A Structure of octanal. B Profile for the fluorescence intensification of FITC-CD36149–168 by the test lipid. Octanal was included in assay mixture within the concentration range indicated. The mean fluorescence of wells exposed to assay mixture containing the lipid at the minimum concentration was set at 1.0. Data are expressed as mean ± S.D. for three independent wells.