The terminal six amino-acids of the carboxy cytoplasmic tail of CD36 contain a functional domain implicated in the binding and capture of oxidized low-density lipoprotein

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CD36, a major adhesion molecule expressed by monocytes/macrophages, plays a key role in the binding and internalization of oxidized low-density lipoprotein (OxLDL). This adhesion molecule, a member of an important scavenger receptor family, contains a very short C-terminal cytoplasmic tail that is known to induce intracellular signalling events. However, the domains on the cytoplasmic tail involved in such signal transduction are unknown. In this study, we have investigated the functional components of the cytoplasmic tail by site-directed mutagenesis coupled with functional OxLDL and monoclonal antibody (mAb) binding studies. Seven truncated or punctual CD36 constructs, localized in the cytoplasmic tail, were produced by site-directed mutagenesis. Each construct was stably expressed in HEK293 cells. We used a quantitative and a qualitative method, labelling OxLDL with either iodine or rhodamine, to determine the functional importance of the cytoplasmic domains in OxLDL internalization. Results indicate that: (1) a deletion of the last amino-acid (construct K472STOP) significantly reduces, compared with wild-type, the binding, internalization and degradation of OxLDL; (2) truncation of the last six amino-acids (construct R467STOP) significantly reduces OxLDL binding; (3) the above two constructs (K472STOP and R467STOP) showed a reduced rate of OxLDL internalization compared with wild-type; (4) the binding and rate of internalization of an anti-CD36 monoclonal antibody (10/5) was not affected by the above mentioned mutants (K472STOP and R467STOP), compared with wild-type. This study shows, for the first time, a specific site on the CD36 cytoplasmic tail that is critical for the binding, endocytosis and targeting of OxLDL.

Key words: atherosclerosis, foam cells, macrophages, scavenger receptors.

INTRODUCTION

CD36 is a highly glycosylated glycoprotein expressed by monocytes, macrophages, platelets, microvasculature endothelial cells, erythrocyte precursors, mammary epithelial cells and adipocytes [1–7]. This 88 kDa adhesion molecule, member of a family comprising several other gene products, was first shown to be a receptor for thrombospondin and collagen [8,9]. It was then also implicated in the phagocytosis of apoptotic neutrophils in tandem with $\alpha v\beta 3$ [10–12]. The site on CD36 binding to apoptotic neutrophils is localized between amino acids 155 and 183 [11]. CD36 is also involved in the cytoadherence of erythrocytes infected with *Plasmodium falciparum* [13].

More recently, CD36 expressed by monocytes/macrophages was shown to play a major role in the endocytosis of oxidized low-density lipoprotein (OxLDL) and fatty acids [7,14,15]. In our laboratory, we have localized the OxLDL binding site on CD36 to a region encompassing amino acids 155–183 [16]. The uptake of OxLDL by macrophages is a crucial step in the initiation and the development of atherosclerotic lesions. Indeed, CD36 plays a major role in the uptake of OxLDL by macrophages and in their differentiation into foam cells within human atherosclerotic lesions [17,18]. OxLDL and circulating cholesterol also induce an increase in CD36 expression in human monocyte-derived macrophages, and in endothelial cells of the microvasculature of the hearts of mice fed a high-fat diet [19,20]. The critical role of CD36 in monocyte differentiation, as shown by Tontonoz et al. [21] and Nagy et al. [22], is linked to its capacity for OxLDL uptake. OxLDL taken up by CD36 induces the activation of peroxisome proliferator-activated factor receptor γ (PPARγ) and the subsequent lipid accumulation in macrophages [21,22]. Mice with a CD36 deficiency, in a knockout mouse model, show a high plasma level of cholesterol, non-esterified free fatty acids and triacylglycerol. This knockout mouse model strongly confirms the major role of this adhesion molecule in the binding and uptake of OxLDL [23]. Most interestingly, the number of foam cells and the size of vascular lesions were greatly reduced in a double knockout of ApoE$^{-/-}$/CD36$^{-/-}$ compared with animals deficient in ApoE$^{-/-}$ [24]. Indeed, evidence is increasing to show that CD36 expression and its functions may be instrumental in controlling the initiation, perpetuation and progression of vascular lesions.

Evidence is also available to show that CD36 is implicated in intracellular signalling events. Ligands, such as thrombospondin, collagen, or monoclonal antibodies (mAbs), binding to CD36 can induce platelet activation and superoxide anion production in monocytes [25–27]. Thrombospondin, in a similar manner to signal transducing multimeric ligands, such as tumour necrosis factor (TNF) or interferon γ, has the capacity to induce a dimerization of membrane expressed CD36 [28–30].This ligand-induced receptor dimerization is an important step in events leading to signal transduction. Interestingly, the cyto-

Abbreviations used: LDL, low-density lipoprotein; OxLDL, oxidized LDL; PPARγ, peroxisome proliferator-activated factor receptor γ; mAb, monoclonal antibody; NF-κB, nuclear factor κB; MEM, minimal essential medium; TNF, tumour necrosis factor; IL, interleukin.

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plasmic tail of CD36 is associated with protein tyrosine kinases of the Src family (Yes, Fyn, Lyn) in platelets and in human dermal microvascular endothelial cells [31,32]. However, the nature of the CD36-protein kinase association and the mechanisms by which CD36 may function in ligand binding and in signal transduction remain to be elucidated. Recently, a study by Lipsky et al. [33] has shown that the C-terminal cytoplasmic domain of CD36 is required for OxLDL modulation of nuclear factor κB (NF-κB) activity.

In the present study, we have investigated the functional components of the cytoplasmic tail by site-directed mutagenesis coupled with functional OxLDL binding studies. Results obtained show, for the first time, a specific site on the CD36 cytoplasmic tail that is critical for the binding and capture of OxLDL.

Table 1 CD36 constructs

Representation of CD36 human constructs localized in the C-terminal cytoplasmic tail. Bold letters indicate the amino acids replaced.

CD36 names	Amino acid sequence
Wild-type	NH_2-GCDRNC----------SYCACRSKTIK-COOH
R467STOP	NH_2-GCDRNC----------SYCAC-COOH
K472STOP	NH_2-GCDRNC----------SYCACRSKTI-COOH
Y463A	NH_2-GCDRNC----------S**A**CACRSKTIK-COOH
C464A	NH_2-GCDRNC----------SY**A**ACRSKTIK-COOH
C466A	NH_2-GCDRNC----------SYCA**A**RSKTIK-COOH
C464-466A	NH_2-GCDRNC----------SY**AA**RSKTIK-COOH
T470A	NH_2-GCDRNC----------SYCACRSK**A**IK-COOH
	| | |
Amino acid no:	1 462 472

MATERIALS AND METHODS

Cell culture

Human epithelial kidney 293 cells (HEK293) were maintained in minimal essential medium (MEM; Life Technologies, Paisley, Renfrewshire, Scotland, U.K.) supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 10 μg/ml streptomycin and 10 units/ml penicillin in 5% CO_2 at 37 °C.

Iodination of anti-CD36 mAb

An anti-CD36 mAb (10/5) was labelled with $Na^{125}I$ using IODO-BEADS reagent (Pierce, Rockford, IL, U.S.A.). Briefly, mAb 10/5 (300 μg) was labelled with 1 mCi of $Na^{125}I$ using two IODO-BEADS, for 15 min at room temperature (20–25 °C). ^{125}I-labelled mAb was isolated using a G-25 Sephadex column (column PD-10, Amersham Pharmacia Biotech, Little Chalfont, Bucks., U.K.). Specific radioactivity was 900–2400 c.p.m./ng of mAb.

Isolation, modification and labelling of OxLDL

Low density lipoproteins (LDLs) were isolated from normolipidaemic human plasma (Transfusion Center, Rungis, France) using a single-step discontinuous gradient in a Beckman NVT65 rotor [34]. Protein content was determined using the BCA assay kit (Pierce). Prior to chemical modification, LDL were dialysed against PBS (pH 7.4). Copper-oxidized LDL were prepared under sterile conditions by incubating 500 μg/ml LDL with 2.5 μM $CuCl_2$ for 48 h at 37 °C. At the end of the incubation period, OxLDL were extensively dialysed at 4 °C against PBS (pH 7.4). OxLDL were characterized by measuring thiobarbituric acid reactive substances (TBARS: 40 ± 5 nmol equivalent malondialdehyde/mg of LDL protein) and lipoperoxides (259 ± 90 nmol/mg of LDL protein) [35,36]. Labelling of OxLDL with ^{125}I was prepared using the IODO-GEN reagent (Pierce). Specific radioactivity, determined for each experiment, ranged from 200 to 400 c.p.m./ng of OxLDL. OxLDL was also labelled with rhodamine succinimidyl ester (Molecular Probes, Europe BV, Leiden, The Netherlands) as described Stanton et al. [37]. All samples were filtered through 0.4 μm Millipore filters.

DNA constructs and transfection

Human CD36 cDNA was kindly provided by Dr Brian Seed (Massachusetts General Hospital, Department of Molecular Biology, Boston, MA, U.S.A.). CD36 cDNA was cloned into the *Hin*dIII and *Xba*I sites of M13mp19. Alanine substitutions or stop codons were introduced into the cDNA sequence by oligonucleotide-directed mutagenesis [38]. In each case, the codon degeneracy was exploited to introduce a novel restriction-endonuclease site to allow rapid identification of mutants without altering the coding sequence [39]. The ligation products were amplified in *Escherichia coli* MC1061p3 and purified on a CsCl gradient. All mutations were confirmed by DNA sequencing. CD36 cDNA constructs were cloned into the *Xba*I sites of a stable expression vector, pCDNA3.1. Each CD36 cDNA construct is represented in Table 1. DNA transfection was performed in HEK293 cells using LIPOFECTAMINE™ (Life Technologies) for 2 h at 37 °C. The cells were selected 48 h after transfection in medium containing 500 μg/ml G418. Each clone was purified by anti-CD36 mAb (10/5) coupled with magnetic beads (Dynal SA, Compiègne, France).

Western blotting

Confluent cells, in a 25 cm² flask, were detached by trypsin/EDTA, washed twice in PBS, pH 7.4, then lysed into 200 μl of 0.02 M Tris/HCl, pH 7, 0.15 M NaCl, containing anti-proteases and 1% (w/v) Lubrol and rocked for 25 min at 4 °C. Lysates was then purified by centrifugation. Cell lysates were separated on an SDS/10% polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane and blocked for 4 h at room temperature (20–25 °C) in blocking buffer [Tris-buffered saline, pH 7.6, 0.05% Tween 20 and 5% (w/v) non-fat dry milk]. After an overnight incubation at 4 °C with primary antibody (10/5 at 10 μg/ml), the blot was incubated with an appropriate secondary anti-IgG-horseradish-peroxidase conjugate. The membrane was washed three times for 30 min in Tris-buffered saline, 0.05% Tween 20, and developed with ECL® chemiluminescent substrate (Amersham).

Flow cytometry

Cells (1×10^6 cells/ml) were washed twice with PBS (pH 7.4), then incubated with the anti-CD36 mAbs (10/5 or 13/10) for 1 h at 4 °C (10 μg/ml) in PBS containing 3% (w/v) BSA. mAbs 10/5 and 13/10, directed against CD36, were produced and characterized in our laboratory [40]. After one wash in PBS, cells were incubated with goat anti-mouse-IgG–FITC conjugate (Dako, Glostrup, Denmark) for 45 min at 4 °C. Cells were washed once in PBS then fixed with 1% (v/v) formaldehyde. The expression level of CD36 and its variants was assessed by flow cytometry using a Becton-Dickinson Facscan and LysisII soft-

ware. The number of CD36 sites of each stable transfected cell line was also quantified in each experiment of binding, degradation and internalization, with fluorescent microbeads using Dako Qifikit (Dako).

Assay for OxLDL rhodamine endocytosis

Cells (2×10^5) of each stably transfected cell line were placed on glass coverslips in 35-mm diameter Petri dishes. After 72 h, cells were incubated with 10 μg/ml rhodamine-OxLDL for 30 min at 37 °C. After three washes in PBS, cells were fixed with 3 % (v/v) paraformaldehyde for 20 min. After two further washes in PBS, cells were mounted in fluorescent mounting medium (Dako).

Binding of mAb and OxLDL

Cells (2×10^5) of the different stably transfected cell line were placed in 35-mm diameter Petri dishes for 72 h. Cells were incubated with different concentrations of ^{125}I-mAb (0.1, 0.25, 0.5, 0.75, 1, 1.5, 2 and 4 μg/ml) or different concentrations of ^{125}I-OxLDL (2, 5, 10, 20 and 40 μg/ml) in the presence or absence respectively of a 10-fold excess of unlabelled mAb and a 15-fold excess of unlabelled OxLDL for 1 h at 4 °C. Cells were then washed twice with PBS containing 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 1 % BSA and twice with PBS containing 0.5 mM $CaCl_2$ and 0.5 mM $MgCl_2$. Washed cells were then dissolved in 0.1 M NaOH and scraped off the plates. An aliquot was used to assay for radioactivity and protein content.

Binding, internalization and degradation of OxLDL

Cells (2×10^5) of each stably transfected cell line were placed in 35-mm diameter Petri dishes for 72 h. Cells were incubated with 10 μg/ml ^{125}I-OxLDL in the presence or absence of a 40-fold excess of unlabelled OxLDL for 4 h at 37 °C. After these incubations at 37 °C, OxLDL proteolytic degradation was measured as trichloroacetic acid- and silver nitrate-soluble radioactivity released into the medium. Cells were washed twice with PBS containing 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 0.2 % BSA and twice with PBS containing 0.5 mM $CaCl_2$ and 0.5 mM $MgCl_2$. Washed cells were then dissolved in 0.1 M NaOH and scraped off the plates. OxLDL binding and internalization was measured as radioactivity contained in the solubilized cell pellets. Protein concentrations was also measured using an aliquot of solubilized cell pellets as described below.

Rate of ligand internalization in HEK293 cells

The assay, previously described in detail by Setiadi et al. [41], is based on the dissociation of surface bound ligands from the cell surface. Indeed, saturating concentrations of ^{125}I-10/5 mAb (2 μg/ml) were incubated with transfected HEK293 cells (30 min, 4 °C) at 60–70 % of confluence. Unbound antibody was washed away and the cells were treated with 0.1 M acetate buffer containing 0.15 M NaCl, pH 4, for 20 min at 37 °C. This treatment removed 40 % of the bound antibody. An identical approach was used to release bound ^{125}I-OxLDL, used at a concentration of 20 μg/ml, and observed to dissociate 30 % cell-surface bound OxLDL. Briefly, the assay was initiated by placing 12-well plates containing transfected HEK293 cells at 60–70 % of confluence at 4 °C for 5 min and then washing once with serum free ice-cold MEM. Medium (500 μl) containing ^{125}I-10/5 mAb (2 μg/ml) were added to each well. After 30 min at 4 °C, cells were rapidly washed twice with ice-cold MEM containing 1 % BSA and twice with ice-cold MEM. For the zero time point, 1 ml of ice-cold MEM was added, incubated for 2 min at 4 °C and then the medium containing spontaneously released ^{125}I-10/5 mAb was collected and counted for radioactivity. This step was immediately followed by the addition of 1 ml of pre-warmed acetate buffer, to release surface-bound mAb, which was left incubating at 37 °C for 20 min and collected for counting. Cells were solubilized with 1 ml of 1 M NaOH and counted. For other time points, the assay was performed by incubating 1 ml of MEM with the HEK293 cells, for 1–15 min, at 37 °C. An identical approach was used for ^{125}I-OxLDL (20 μg/ml) with the exception that the time points ranged from to 0 to 3 min. Calculation of the percentage internalization, release and cell-surface bound ligand was identical to the procedure used by Setiadi et al. [41].

Protein measurement

Protein concentrations were determined by the method of Lowry et al. [42], with BSA as the standard, using a Micro Protein Determination kit (Sigma, St Quentin-Fallavier, France).

Statistics

Data presented are means $\pm$ S.D. Differences were analysed by Student's t test. A level of $P < 0.05$ was accepted as statistically significant.

RESULTS

Stable expression of CD36 in HEK293 cells

HEK293 cells were transfected with a stable expression vector bearing different CD36 constructs, having either punctual mutations or deletions, localized in the C-terminal cytoplasmic domain of CD36 (Table 1). Surface expression of the different CD36 constructs in HEK293 cells was assessed by flow cytometry using anti-CD36 mAbs 10/5 (Figure 1A) and 13/10 (results not shown). mAb10/5 recognized the different CD36-transfected, but not the non-transfected, HEK293 cells. Heterogeneous expression between the different transfected cell lines was observed. Quantification of surface CD36 expression, for comparison between different cell constructs and ensuing endocytosis, was performed using Dako Qifikit (results not shown). Stably transfected (CD36wt, R467STOP and K472STOP), but not non-transfected, HEK293 cells showed an 88 kDa band by Western blot analysis which represents the protein of the CD36 wild-type construct (Figure 1B). The five other constructs of CD36 have been also characterized by Western blot analysis and they also showed a band recognized by mAb 10/5, at the same molecular mass as the wild-type (results not shown).

Binding and endocytosis of rhodamine-OxLDL

OxLDL labelled with rhodamine, bound and endocytosed via the different CD36 constructs expressed by HEK293 cells, was localized at the cellular membrane level and in cytoplasmic vesicles (Figure 2A). However, the extent of binding and internalization of rhodamine-OxLDL was dependent upon the type of construct. Constructs R467STOP and K472STOP, unlike the wild-type or other constructs, showed decreased binding and internalization of OxLDL (Figure 2A and results not shown).

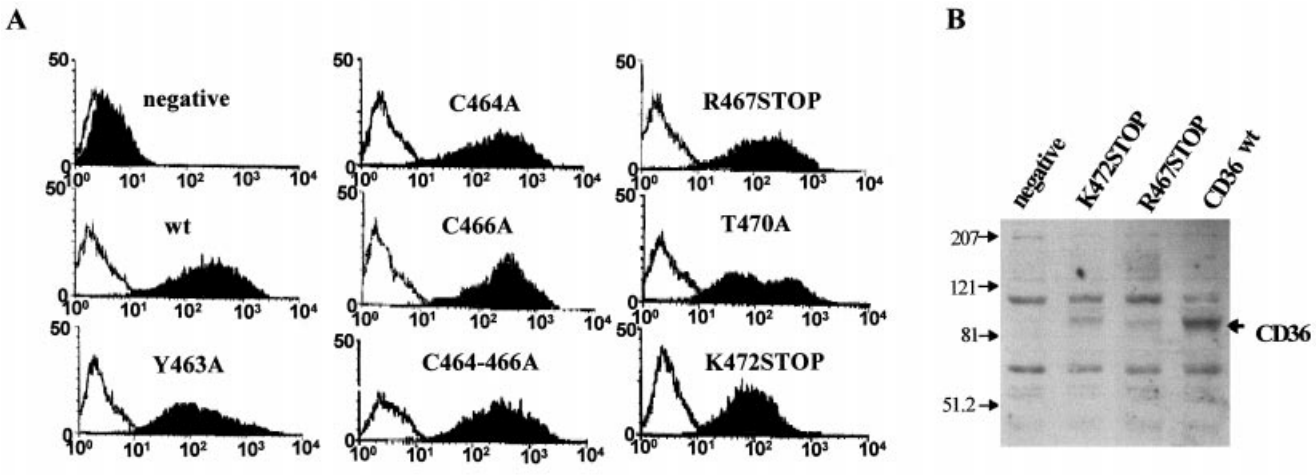

Figure 1 Characterization of CD36 stably transfected HEK293 cell lines

(**A**) Flow cytometry analysis of CD36-transfected cells and control HEK293 cells in the absence or presence of anti-CD36 mAb 10/5 for each CD36 construct. HEK293 cells were detached, washed, incubated with 5 μg/ml anti-CD36 mAb 10/5 for 1 h at 4 °C, washed, then incubated with anti-mouse FITC antibody for 45 min at 4 °C, and then resuspended in PBS and fixed with 1% formaldehyde and analysed in the FACScan with LysisII software. (**B**) Western blot analysis of cell lysates from transfected CD36 constructs. Cell lysate proteins were separated by SDS/PAGE (50 μg of protein), transferred to nitrocellulose, probed with 10 μg/ml anti-CD36 mAb 10/5, and detected by chemiluminescence. Lane 1, non-transfected HEK293 cells; lane 2, CD36 K472STOP-transfected HEK293 cells; lane 3, CD36 R467STOP-transfected HEK293 cells; lane 4, CD36 wild-type-transfected HEK293 cells. Molecular-mass markers (kDa) are shown on the left.

Binding, internalization and degradation of ^{125}I-OxLDL

Affinity and time courses for OxLDL, at 37 °C for CD36 transfected HEK cells, showed that the saturating concentration of ^{125}I-OxLDL at 37 °C for the transfected cells was 10 μg/ml [14]. ^{125}I-OxLDL at 10 μg/ml was used for binding, internalization and degradation studies to characterize the different CD36 constructs expressed by HEK293 cells. Two constructs (R467STOP and K472STOP), with partial deletions of the C-terminal cytoplasmic tail, showed significant decreases in binding and internalization (81.2% and 57.2% respectively; $P < 0.01$), and degradation (85.5% and 61.2% respectively; $P < 0.05$) of ^{125}I-OxLDL (10 μg/ml) compared with wild-type CD36 (Figures 2B and 2C). These data suggest a major role for the CD36 C-terminal cytoplasmic tail in OxLDL endocytosis. Several other punctual constructs were performed on the cytoplasmic tail. Constructs mutating cysteine to alanine (C464A, C466A and C464–466A) did not affect the endocytosis of OxLDL (Figures 2B and 2C). This would tend to suggest that the conformation of the C-terminal cytoplasmic tail is not required for OxLDL endocytosis. Moreover, two further punctual constructs of amino acids that could possibly be implicated in signal transduction (Y463A and T470A) did not affect OxLDL uptake and degradation (Figures 2B and 2C).

^{125}I-OxLDL affinity to CD36 constructs

Scatchard analysis was used to investigate the affinity of OxLDL to the wild-type and the different CD36 constructs. Increasing concentrations of ^{125}I-OxLDL (2, 5, 10, 20 and 40 μg/ml) were added in the presence, or absence, of excess unlabelled OxLDL, to the wild-type and to different CD36 constructs expressed by HEK293 cells. Following correction for the binding of OxLDL to non-transfected cells (Figures 3A and 3B), the maximal-binding data presented in Table 2, showed heterogeneity of CD36 expression between the cell lines. Such heterogeneity is in line with results obtained from corresponding flow cytometry experiments (results not shown). Wild-type and constructs, with the exception of R467STOP, did not show a difference in maximal binding of OxLDL ($n = 3$). Maximal binding of OxLDL to R467STOP was lower than that observed for the wild-type and other constructs. The dissociation constant (K_d) of the different CD36 constructs, after correction for the contribution of non-transfected HEK293 cells, was obtained via Scatchard analysis (Table 2). The K_d for all constructs, except R467STOP, showed no significant difference (Table 2).

Measurement of binding and internalization of mAb 10/5 in HEK293 cells

Having identified two constructs showing different binding, capture and degradation of OxLDL, we were interested to see what effect, if any, it had on the affinity and internalization of a functional anti-CD36 mAb. Hence, affinity of mAb 10/5 to CD36 wild-type and two constructs (R467STOP and K472STOP) expressed in HEK293 cells was performed. Results, presented in Figure 4, show no difference in K_d between wild-type and mutated transfected constructs (R467STOP and K472STOP). However, the maximal-binding values, obtained in these experiments, is lower for R467STOP and K472STOP than that observed for the wild-type. Such difference in binding is probably due to the heterogeneity of CD36 expression between cell lines, as indicated by corresponding flow cytometry analysis (results not shown). Hence the affinity of mAb 10/5, a functional blocking mAb known to be directed against a site on CD36 binding OxLDL, or the weak internalization (results not shown) is not altered by the deletion introduced in the C-terminal cytoplasmic tail.

Measurement of internalization of OxLDL in HEK293 cells

To further confirm the data presented in Figure 2, we assayed the rate of internalization of OxLDL by the wild-type and the two constructs of interest (R467STOP and K472STOP) expressed

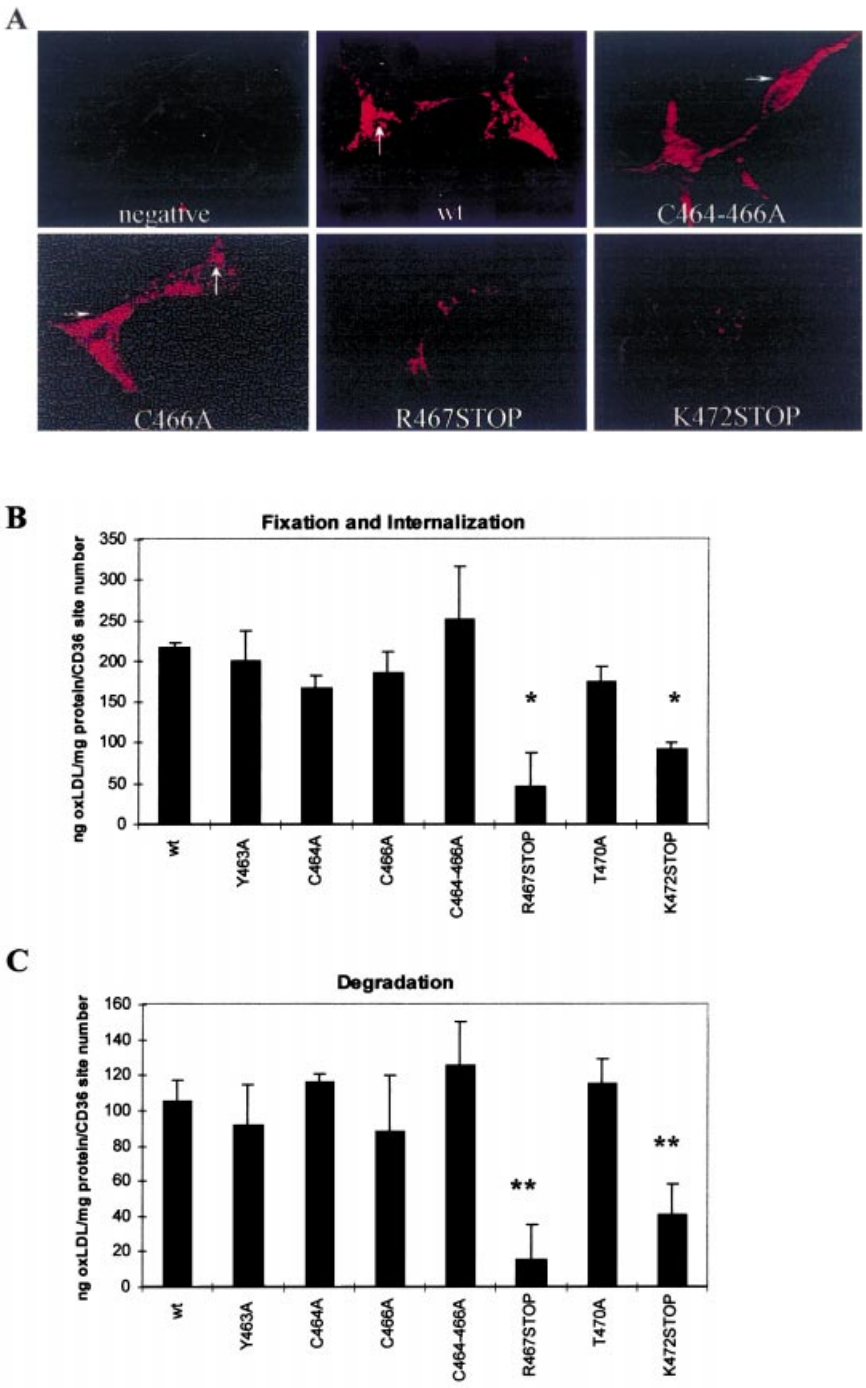

Figure 2 OxLDL uptake by CD36-transfected HEK293 cell lines

(**A**) Rhodamine-OxLDL endocytosis by CD36 constructs. Cells plated on glass coverslips were incubated with 10 μg/ml rhodamine-OxLDL for 30 min at 37 °C. Cells were then washed three times, fixed in 4% paraformaldehyde and mounted in fluorescent mounting medium. Rhodamine-OxLDL endocytosis by constructs not shown in this Figure, were similar to the result obtained for CD36 wild-type. Vertical arrows show the OxLDL uptake. Horizontal arrows show the OxLDL bound at the cell surface. (**B**) and (**C**) ^{125}I-OxLDL fixation, internalization and degradation by CD36 constructs. Stable CD36 transfections were incubated with 10 μg/ml ^{125}I-OxLDL for 4 h at 37 °C in the absence or presence of a 40-fold excess of Ox-LDL, to measure non-specific binding. Values represent the specific binding and internalization (**B**) or degradation (**C**) for three independent experiments in triplicate wells (mean $\pm$ S.D.) after correction for the contribution from non-transfected HEK293 cells. The data are expressed as ng of OxLDL/mg of protein per number of CD36 sites (ng oxLD/mg protein/CD36 site number). $^{*}P < 0.01$, $^{**}P < 0.05$ compared with human wild-type CD36.

by HEK293 cells. In the CD36 wild-type, in contrast to the two constructs, the rate of internalization of bound OxLDL reached a plateau after 1 min, at which 30% of total bound OxLDL was endocytosed (Figure 5). Spontaneous release of ^{125}I-OxLDL, bound to the wild-type CD36-transfected HEK293 cells, was observed to be at 35% and was maintained at that level at all time points (results not shown). Construct R467STOP, at 1 min, internalized approx. 10% of total bound

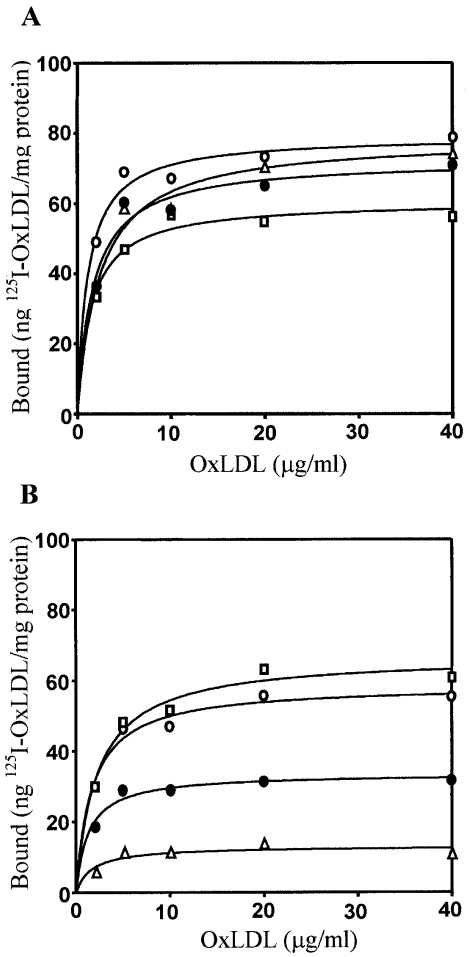

Figure 3 Binding of ^{125}I-OxLDL to CD36-transfected HEK293 cell lines

Cells were incubated with ^{125}I-OxLDL at different concentrations (2, 5, 10, 20 and 40 μg/ml) for 1 h at 4 °C in the presence or absence of a 15-fold excess of OxLDL. Cells were washed twice in PBS containing 1% BSA and twice in PBS, the bound radioactivity was counted after solubilizing the well contents in 0.1 M NaOH, and an aliquot was used to measure the protein concentration. Each point represents the mean for three separate experiments after correction for non-specific values and non-transfected HEK293 cell values. (**A**) CD36 wild-type (○), Y463A (□), C464A (△) and C466A (●). (**B**) C464–466A (○), T470A (□), R467STOP (△) and K472STOP (●).

Table 2 Dissociation constants and maximal-binding values of OxLDL for CD36 constructs

OxLDL dissociation constants (K_d) for CD36 constructs, after correction for non-specific values and for the contribution by non-transfected HEK293 cells, were obtained by Scatchard analysis. The maximal-binding values were obtained by OxLDL binding graphic analysis. Means of K_d and OxLDL maximal-binding values were obtained from three separate experiments and three different preparations of OxLDL. $^*P < 0.05$ K_d and maximal-binding values of R467STOP is significantly different from wild-type CD36.

CD36 names	K_d (μg/ml)	Maximal-binding values (ng of OxLDL/mg of protein)
Wild-type	3.16 ± 1.73	78.92 ± 43.20
R467STOP	7.98 ± 2.84*	12.92 ± 4.60*
K472STOP	4.01 ± 0.66	33.49 ± 5.51
Y463A	2.91 ± 0.89	78.35 ± 23.96
C464A	4.39 ± 1.24	72.19 ± 20.39
C466A	3.53 ± 0.93	60.43 ± 15.92
C464-466A	3.59 ± 1.10	58.45 ± 17.85
T470A	3.52 ± 1.03	66.66 ± 19.50

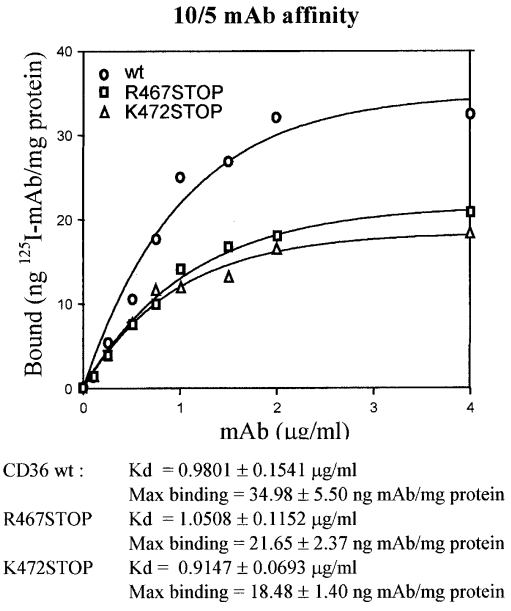

Figure 4 Binding of ^{125}I-mAb 10/5 to CD36-transfected HEK293 cell lines

Cells were incubated with ^{125}I-mAb 10/5 at various concentrations (0.1, 0.25, 0.5, 0.75, 1, 1.5, 2 or 4 μg/ml) for 1 h at 4 °C, in the presence or absence of a 10-fold excess of mAb 10/5. Cells were then washed twice with PBS containing 1% BSA and twice with PBS, then bound radioactivity was counted after solubilizing the well contents in 0.1 M NaOH, an aliquot was also used to measure the protein content. Each point represents the mean for four separate experiments. CD36 wild-type (CD36 wt; ○), R467STOP (□) and K472STOP (△).

OxLDL and no plateau was reached even at 3 min (Figure 5). The other construct, K472STOP, at 1 min showed around 15% internalization of total bound OxLDL and a plateau at 2 min (Figure 5). Spontaneous release of ^{125}I-OxLDL, bound to R467STOP and K472STOP constructs expressed in HEK293 cells, was observed to be at 40% and was maintained at that level at all time points (results not shown).

DISCUSSION

This study identifies, for the first time, specific sites on the CD36 cytoplasmic tail that are involved in the binding, uptake and degradation of OxLDL. Several lines of evidence clearly implicate specific sites of the CD36 cytoplasmic tail in this crucial scavenging activity. (1) A deletion of the last amino-acid (construct K472STOP) significantly reduces, compared with wild-type, the binding, internalization and degradation of OxLDL. (2) Truncation of the last six amino-acids (construct R467STOP) significantly reduces OxLDL binding. (3) These two constructs (K472STOP and R467STOP) showed reduced rates of OxLDL internalization compared with wild-type. (4) Binding and the rate of internalization of an anti-CD36 mAb (10/5) was not affected, compared with wild-type, by these mutations (K472STOP and R467STOP).

CD36 has been shown to bear palmitoylated N- and C-terminal cytoplasmic tails [43]. The presence of palmitoylated cysteine residues strongly suggests the presence of two intracellular domains. Moreover, assignment of disulphide bridges in

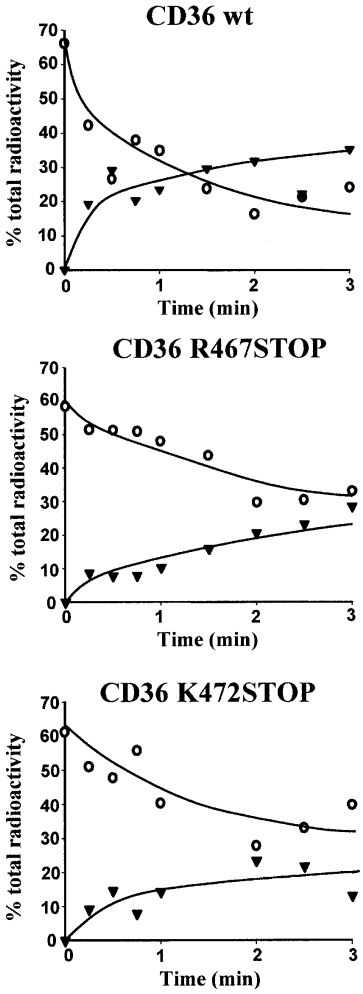

Figure 5 Rate of internalization of ^{125}I-OxLDL by CD36-transfected HEK293 cell lines

Cells were incubated with ^{125}I-OxLDL at 20 μg/ml for 30 min at 4 °C. They were then twice washed with ice-cold MEM containing 1 % BSA and twice with ice-cold MEM. The internalization of OxLDL by CD36 wild-type (top panel) and constructs R467STOP (middle panel) and K472STOP (bottom panel) was measured by the removal of ^{125}I-OxLDL by an acidic buffer as described in the Material and methods section. Internalized ^{125}I-OxLDL (▼), cell surface ^{125}I-OxLDL (○). Each point represents the mean of one experiment in duplicate.

bovine CD36 suggests that the bovine CD36 contains two short (residues 1–6 and 461–471) intracellular and transmembrane domains [44]. In contrast, two other results suggest the possibility of only one cytoplasmic domain located at the C-terminal end of CD36 [30,45]. In these studies, the authors have truncated the CD36 protein by removing the intracytoplasmic and transmembrane domains (a deletion of 43 amino acids), and this resulted in a secreted form of CD36. Moreover, analysis of the proposed N- and C-terminal domain shows that only the C-terminal domain has a potential capacity to induce signal transduction by bearing amino acids capable of being phosphorylated and becoming binding sites for a variety of intracellular molecules. In the present study, we have produced two CD36 mutants (constructs R467STOP and K472STOP), bearing truncated C-terminal cytoplasmic tails, that very significantly affect CD36 functions. Our results argue for a determining role of the C-terminal tail in the control of CD36 functions. Moreover, such results complement those obtained by Lipsky et al. [33], who showed that a domain at the C-terminal end of CD36 (the last 6 amino acids) was implicated in the activation of NF-κB following tumour necrosis factor (TNF) α and OxLDL activation.

A significant advance in the elucidation of the mechanism by which CD36-mediates signal transduction was reported by Huang et al. [31] and Bull et al. [32]. In these studies, the authors demonstrated that the cytoplasmic tail of CD36 is associated with pp60^{fyn}, pp62^{yes} and pp54/58^{lyn} protein tyrosine kinases of the *src* gene family in platelets, human C32 melanoma, human erythroleukaemia cells and human dermal microvascular endothelial cells. However, the nature of the association between the cytoplasmic protein tyrosine kinases and CD36 has yet to be clearly defined. mAb or ligand-induced receptor oligomerization is an important step in the signal transduction pathway. Studies have shown that CD36 can exist in a dimer form following ligand binding [30,46]. F(ab′)2 fragments of a CD36 mAb (OKM5) activate platelets, and induce an oxidative burst and the generation of superoxide anion in monocytes [26,27]. Moreover, activation of human monocytes by an anti-CD36 mAb initiates a cellular signalling pathway characterized by an intracellular tyrosine phosphorylation as well as extracellular signal-regulated kinase ('ERK') and p38 mitogen-activated protein kinase ('MAPK') phosphorylation [47]. OxLDL binding to CD36 is implicated in the activation of transcriptional factors such as NF-κB factor and the nuclear receptor PPARγ [21,33]. It is to be noted that, in human macrophages expressing CD36 but not in CD36-deficient macrophages, activation of NF-κB by OxLDL induces the expression of several cytokines such as interleukin (IL)-1Ra, IL-1β, IL-6, TNFα, TNFβ, interferon γ and interferon β, implicated in the development of atherosclerosis [48].

Such an activation, hypothetically implicating clustering of CD36 by OxLDL, allows differentiation and an increased expression of CD36 synthesis on monocytes/macrophages. Tontonoz et al. [21] also showed an increase in CD36 expression mediated by the nuclear hormone receptor heterodimer, PPARγ/retinoid X receptor α. Recently, the signalling pathway leading to PPARγ activation and CD36 over-expression has been partially determined and implicates the protein kinase C pathway [49]. In fact, treatment of murine macrophages by protein kinase C inhibitors, following OxLDL stimulation, inhibits the activation and induction of PPARγ and enhanced CD36 expression.

Previous study on the cytoplasmic tail of P-selectin, using anti-P-selectin mAb, showed that this domain controlled the endocytosis of this adhesion molecule in transfected Chinese hamster ovary cells [41]. In the present study, the two constructs (K472STOP and R467STOP) that affected CD36 functions with regards to the binding and endocytosis of OxLDL had no equivalent effect on anti-CD36 mAb (10/5). The mAb used in this study has been shown previously to be directed against one of the two identified sites for OxLDL binding on CD36 [16,50]. It is conceivable that the two constructs affect the delicate mechanism necessary to endocytose quite large molecules, such as OxLDL, and have no visible effect on the mAbs used.

In summary, this study shows that a specific site in the C-terminal cytoplasmic domain of CD36 is crucial for the binding, internalization and degradation of OxLDL. This cytoplasmic domain has previously been implicated in NF-κB activation, which subsequently induced CD36 synthesis [19,33]. In conclusion, our results allow a better understanding of the role of CD36 in the mechanism controlling OxLDL binding and endocytosis. This should eventually lead to novel methods to prevent

the progression and the development of atherosclerosis in human arterial walls.

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