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THE CD36 AMINO-CYTOPLASMIC DOMAIN IS NOT REQUIRED FOR THE INTERNALISATION OF OXIDISED LOW DENSITY LIPOPROTEIN

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Abbreviations: OxLDL, Oxidised low density lipoprotein; TSP-1, Thrombospondin-1; FITC, fluorescein isothiocyanate; DiI, 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate; PNGase F, Peptide-N-glycosidase F

ABSTRACT

The uptake of oxidised low density lipoproteins (OxLDL) by CD36-expressing macrophages in the arterial intima and the ensuing 'foam cell' formation represents a crucial step in the initiation and development of atherosclerotic plaques. This study has addressed the function of the CD36 amino-cytoplasmic domain in the binding and internalisation of OxLDL. A selection of CD36 amino-cytoplasmic domain mutants were generated and stably expressed in HEK293 cells. The capacity of three mutants (CD36_C3/7-A, CD36_D4/R5-A and CD36_nCPD-) to bind and endocytose OxLDL was then studied using immunofluorescence microscopy and quantitative fluoremetry. Each of the CD36 constructs was expressed at differing levels at the cell surface as measured by flow cytometry and western blotting. Following incubation with DiI-OxLDL, cells bearing the CD36_wild-type, CD36_C3/7-A, CD36_D4/R5-A and CD36_nCPD- construct all internalized DiI-OxLDL into endosomal structures whereas empty vector-transfected cells failed to do so indicating that unlike the carboxy-cytoplasmic domain, the amino-cytoplasmic domain is not essential for the endocytosis of OxLDL. To conclude, the uptake of OxLDL by CD36 is not reliant on the presence of the CD36 amino-cytoplasmic domain. However, the amino-cytoplasmic domain may conceivably be implicated in the maturation of CD36.

INTRODUCTION

The uptake of oxidatively-modified low density lipoproteins (OxLDL) by macrophages in the arterial intima and the ensuing 'foam cell' formation represents a crucial step in the initiation and development of atherosclerotic plaques. Uptake of OxLDL is mediated by certain members of the scavenger receptor family, a group of widely expressed, multi-functional membrane-embedded glycoproteins. A major receptor for OxLDL is CD36 [1] and evidence implicates it as a key-player in foam cell formation and atherosclerotic lesion development. For example, macrophages obtained from CD36-deficient individuals bind and endocytose OxLDL far less effectively than those from normal subjects [2] and CD36-deficient macrophages are impaired in their capacity to secrete pro-inflammatory cytokines following OxLDL treatment [3], a hallmark event of atherogenesis. Furthermore, CD36-ApoE double-null mice develop markedly smaller atherosclerotic plaques than ApoE^{-/-} mice [4].

Based on findings by the likes of Gruarin et al. [5] and Tao et al. [6], CD36 is predicted to adopt a ditopic configuration meaning it comprises an extracellular domain (residues 29-438) flanked by two transmembrane domains (residues 7-28 and 439-460) and two cytoplasmic domains (residues 1-6 and 461-472). A 28 residue segment in the extracellular domain (155-183) has been found to mediate binding with OxLDL [7]. Interestingly, despite the compartmental separation between the extracellular domain and the carboxy-cytoplasmic domain, the latter has been found to be essential for the binding and uptake of OxLDL as well as signal transduction processes. Our group previously demonstrated that HEK293 cells expressing a construct lacking the last 6 residues (467-472) of the carboxy-cytoplasmic domain displayed an approximate 80 % decrease in their capacity to bind and internalise OxLDL. The carboxy-cytoplasmic domain also possesses signal transduction properties; Lipsky et al. reported a significant decrease in OxLDL-mediated NF- κ B activation in CHO cells expressing a CD36 construct lacking the carboxy-cytoplasmic domain compared to cells bearing the wild-type protein [8]. Similarly, the anti-angiogenic effect of TSP-1 observed in CD36-transfected endothelial cells is abrogated if certain residues in the carboxy-cytoplasmic domain (Cysteine 464, Arginine 467, and Lysine 469) are mutated [9].

To our knowledge, the CD36 amino-cytoplasmic domain has not been investigated with regards to its involvement in ligand binding and uptake. The oppositely charged aspartate – arginine sequence at positions 4 and 5, respectively may provide a means for CD36 to interact with co-receptors and / or facilitate capping and endocytosis of CD36-ligand complexes.

Similarly, the palmitoyl group shown to be appended to each of the cysteine residues at positions 3 and 7 [5] may mediate an interaction with membrane or cytoplasmic proteins that is necessary for CD36 binding and endocytosis. Hence, to investigate the role of the amino-cytoplasmic domain in the binding and internalisation of OxLDL, a selection of CD36 amino-cytoplasmic domain mutants were generated and stably expressed in HEK293 cells. The capacity of these mutants to bind and endocytose OxLDL was studied using immunofluorescence microscopy and quantitative fluoremetry.

MATERIALS AND METHODS

Cell culture

Human epithelial kidney 293 (HEK293) cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, 10 µg/ml streptomycin and 10 units/ml penicillin in a 5 % CO₂, 95 % air atmosphere at 37°C.

Plasmid construction

The CD36 coding sequence was PCR-amplified from an M13mp19 cloning vector containing the CD36 coding sequence using the wild-type forward and reverse primers shown in Table 1 and sub-cloned into the pcDNA3.1(+) expression vector. Three amino-cytoplasmic domain mutant constructs (Figure 1) were generated by PCR-mutation of the wild-type CD36 sequence using the primer sequences shown in Table 1. As with the wild-type construct, all mutated constructs were sub-cloned into the pcDNA3.1(+) expression vector and validated by DNA sequencing.

Stable expression of CD36 constructs in HEK293 cells

Transfection of plasmid DNA into HEK293 cells was carried out using Polyfect reagent (Qiagen) according to the manufacturer's instructions. 48 hours post-transfection, growth medium was removed and replaced with complete medium containing genitacin (500 µg/ml) in order to select for stably-transfected cells.

SDS-PAGE, Western blotting and immunodetection

Protein lysates were prepared in accordance to a Triton X-100 membrane protein-enrichment method. Protein concentration was determined using the bicinchoninic acid assay. SDS-PAGE, Western blotting and immunodetection of CD36 were carried out essentially as described by Malaud et al. [10].

Flow cytometry

Cells (1×10^6) were incubated in PBS containing the mouse anti-human CD36 monoclonal antibody 10.5 (5 µg/ml) (Biocytex) for 1 hour at 4°C with vertical rotation. After a single wash with PBS, cells were incubated in PBS containing a mouse anti-human FITC-conjugated antibody (80 µg/ml) for a further hour at 4°C with vertical rotation. Finally, cells were washed to remove residual antibody, re-suspended in 1 % paraformaldehyde and analysed on a Becton Coulter FC 500 flow cytometer with Cytomics RXP Analysis software.

Visualisation of DiI-OxLDL uptake

HEK293 cells expressing the wild-type CD36 construct as well as the various mutated CD36 constructs were seeded in 6-well plates (400,000 cells/well) and grown on cover-slips for 24 hours before being incubated with 50 µg/ml DiI-OxLDL (in complete media) for 16 hours. Cells were rinsed with PBS, fixed with 4 % paraformaldehyde, mounted on to microscope slides using Vectashield (Vector Laboratories) and viewed at the specified magnification using a fluorescence microscope.

Quantification of DiI-OxLDL uptake

HEK293 cells stably-transfected with plasmids detailed above were seeded in 6-well plates (400,000 cells/well) and cultured for 24 hours before being incubated with 0, 25 and 50 µg/ml DiI-OxLDL (in complete media) for 16 hours at 37°C. Uptake of DiI-OxLDL was quantified according to the method of Luan and Griffiths [11]. Cells were harvested with trypsin/EDTA, washed twice with PBS and lysed into 5 % Triton X-100. DiI-OxLDL fluorescence was measured at 590 nm following excitation at 520 nm using a Molecular Devices SpectraMax Microplate Reader. Fluorescence values were converted to µg DiI-OxLDL via a standard curve produced by measuring the fluorescence at 590 nm of lysis buffer containing 0.1, 0.2, 0.5, 1.0 and 1.5 µg DiI-OxLDL. The protein concentration of each sample was determined via the BCA assay. Finally, the quantity of DiI-OxLDL taken up by cells was calculated as amount of OxLDL per mg of cellular protein.

RESULTS

Expression of CD36 constructs in HEK293 cells

HEK293 cells were stably-transfected with a plasmid encoding the CD36 wild-type sequence as well as plasmids encoding the desired amino-cytoplasmic domain mutant constructs. All constructs were expressed, although at differing levels, as found by Western blotting (Figure 2). Interestingly, the anti-CD36 antibody 10.5 recognised two distinct bands in proteins derived from cells expressing the CD36_{nCPD} construct. These may represent differentially-glycosylated forms of CD36 and therefore would suggest an involvement of the amino cytoplasmic domain region in the maturation process. Each of the four CD36 constructs was expressed at differing levels at the cell surface as measured by flow cytometry (Figure 3a). The C3/7-A construct was marginally more abundant than the wild-type construct whilst the D4/R5-A and the nCPD constructs were approximately twice and three times more abundant, respectively than the wild-type construct (Figure 3b).

Visualisation of DiI-OxLDL uptake by HEK293 cells bearing CD36 mutants

To determine whether the CD36 amino-cytoplasmic domain is required for binding and internalization of OxLDL, the passage of fluorescently-labelled (DiI)-OxLDL was tracked in HEK293 cells stably expressing each of the described CD36 constructs using immunofluorescence microscopy. Following incubation with 10 µg/ml DiI-OxLDL for 16 hours at 37°C, cells bearing the CD36_{wild-type}, CD36_{C3/7-A}, CD36_{D4/R5-A} and CD36_{nCPD} construct all internalized DiI-OxLDL into endosomal structures whereas ev-transfected cells failed to do so (Figure 4) indicating that unlike the carboxy-cytoplasmic domain, the amino-cytoplasmic domain is not essential for the endocytosis of OxLDL.

Quantification of DiI-OxLDL uptake by HEK293 cells bearing CD36 mutants

Despite the apparent expendability of the amino-cytoplasmic domain for OxLDL uptake, it was considered that different constructs may still display variation with respect to their binding and internalization efficacy. In order to compare the relative effectiveness of each construct to bind and internalize OxLDL, cells stably-expressing each construct were incubated with DiI-OxLDL as described in the Materials and Methods section. Each of the cell-types accumulated DiI-OxLDL in a dose-dependent manner, again indicating that the amino-cytoplasmic domain is not essential for OxLDL binding and uptake. HEK293-CD36_{wt} cells accumulated approximately twice as much DiI-OxLDL as ev-transfected control cells but an equal amount to HEK293-CD36_{C3/7-A} cells whilst HEK293-CD36_{D4/R5-A} and HEK293_{nCPD} cells accumulated approximately three and four times more DiI-OxLDL, respectively than ev-transfected control cells (Figure 5a). This variation was thought to be resultant of the different levels of expression of each construct. Indeed, when data was normalized to compensate for the differential

expression of each construct, the efficacy of each cell-type to accumulate DiI-OxLDL is approximately equivalent (Figure 5b).

DISCUSSION

Class B scavenger receptors such as CD36 are composed of an extracellular domain, two trans-membrane domains and two cytoplasmic domains. The carboxy-cytoplasmic domain of CD36 has previously been shown to be crucial in the binding and internalization of OxLDL [10] and the Gram positive bacterium *Staphylococcus aureus* [12], and is also required for TSP-1-induced anti-angiogenic signalling [9] as well as NF- κ B trans-activation [8]. To the best of our knowledge, the role of the CD36 amino-cytoplasmic domain in such events has yet to be addressed.

The amino-cytoplasmic domain of CD36 comprises the following residues: H₂N-Met-Gly-Cys-Asp-Arg-Asn-Cys (where H₂N refers to the amino-most residue). Two regions of this sequence were felt to be of interest. Firstly, the cysteine residues (at positions 3 and 7) are known to carry palmitate groups. Such moieties have previously been demonstrated to mediate protein-protein and protein-lipid interactions. Secondly, the oppositely charged aspartate (-ve)-arginine (+ve) sequence (at positions 3 and 4, respectively) may also permit interactions with other receptor proteins and/or cytosolic proteins. To investigate the role played by the amino-cytoplasmic domain (with specific emphasis on the abovementioned regions) in the expression of CD36 and the internalisation of OxLDL, three different mutants were generated by standard PCR-mutagenesis techniques. In the first mutant (C3/7-A), the two cysteine residues were converted to alanine; in the second mutant (D4/R5-A), the aspartate and arginine residues were converted to alanine; in the third mutant (nCPD⁻), the entire amino-cytoplasmic domain was removed. The three CD36 mutants as well as the wild-type construct were then stably-transfected in HEK293 cells. All constructs (including the wild-type) were synthesized and expressed at the cell surface as determined by flow cytometry, albeit at differing levels; the wild-type and the C3/7-A construct were expressed at the cell membrane at a similar level whereas the D4/R5-A construct and the nCPD⁻ constructs were expressed approximately 2- and 3-fold higher, respectively than the wild-type construct. The fact that the amino-cytoplasmic domain constructs were expressed more highly than the wild-type protein at the cell surface would suggest that this region may suppress the migration of the CD36 protein from the cytoplasm to the plasma membrane, perhaps in order to lengthen its exposure to the various post-translational modification enzymes needed for tertiary structure acquisition and attachment of extra groups such as sugar moieties. Indeed, this may explain why the nCPD⁻ construct seemed to comprise two species when analysed by western blotting; instead of a single band being present at approximately 85 kDa, two bands were present – one at approximately 85 kDa and one at approximately 80-82 kDa. The smaller band may represent an incompletely glycosylated species and would indicate that part of or the entire amino-cytoplasmic domain is required for glycosylation of CD36. Assessment of the effect of removing glycosylation sites in CD36 on its membrane trafficking would be advantageous. It should be noted that in our hands, PNGase F-mediated deglycosylation of wild-type CD36 eliminated recognition by the anti-CD36 monoclonal antibody 10.5 (results not shown) suggesting that the epitope recognised by 10.5 comprises sugar groups as well as protein. Indeed, the region of CD36 thought to become bound by 10.5 holds one N-glycan group.

Immunofluorescence microscopy was next used to track the movement of fluorescently-labelled (DiI)-OxLDL in the transfected HEK293 cells expressing the various CD36 mutants. DiI-OxLDL was consistently observed to accumulate in cells expressing the wild-type CD36 construct as well as the three mutated forms but not in ev-transfected (control) cells following an overnight incubation indicating that the amino-cytoplasmic domain is not essential for OxLDL uptake. A fluorescence-based assay adapted from Luan and Griffiths [11] was utilized to quantify

the uptake of DiI-OxLDL by cells expressing each of the CD36 constructs. Consistent with the immunofluorescence work, cells bearing the different mutants were capable of binding and endocytosing OxLDL. In such analyses, there was a notable difference in the quantity of DiI-OxLDL taken up by each cell type. Cells expressing the wild-type and C3/7-A constructs both internalised approximately two-fold more DiI-OxLDL than ev-transfected cells, whilst cells expressing the D4/R5-A and nCPD⁻ constructs internalised approximately four-fold and five-fold more DiI-OxLDL, respectively than the ev-transfected cells. These differences were felt to be due at least partially to the fact that the different constructs were expressed at different levels at the cell surface of transfectants. As a means of compensating for the disparity in construct expression, all DiI-OxLDL uptake values were normalised against the amount of CD36 expressed by each cell line. After doing this, there was a negligible difference in the efficacy of each cell-type to take up DiI-OxLDL, again suggesting that the CD36 amino-cytoplasmic domain is not involved in the endocytotic mechanism. To conclude, the uptake of OxLDL by CD36 is not reliant on the presence of the CD36 amino cytoplasmic domain. However, this region may somehow be implicated in the maturation of CD36. Specifically, it could be involved in retarding the movement of the CD36 polypeptide – following translation - from the cytoplasm to the plasma membrane in order for it to attain its final, native state structure and receive additional moieties such as sugar chains and palmitoyl groups.

THIS IS NOT THE FINAL VERSION - see doi:10.1042/BSR20080045

FIGURES

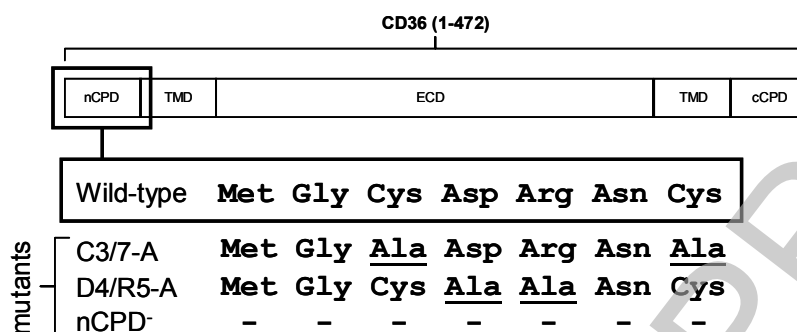


Figure 1: Sequence of generated CD36 amino-cytoplasmic domain mutants. All constructs were generated using primers specified in Table 1 by PCR-mutation and directionally-cloned into the pcDNA3.1(+) expression vector between the *Kpn* I and *Xba* I restriction sites.

Table 1: Primers used for generation of CD36 wild-type and amino-cytoplasmic domain mutant constructs. The italic region in the forward and reverse sequences denote *Kpn* I and *Xba* I restriction sites, respectively.

Construct	Forward (5'-3')	Reverse (5'-3')
Wild-type	ATAGGTACCATGGGGTGTGACCGGAA	TACATTCTAGATTATTTTATTGTTTTCGATC
C3/7-A	AGAGGTACCATGGGCTGTGCCGCAACTGT	TACATTCTAGATTATTTTATTGTTTTCGATC
D4/R5-A	AGAGGTACCATGGGCTGTGCCGCAACTGT	TACATTCTAGATTATTTTATTGTTTTCGATC
nCPD ⁻	ATAGGTACCATGGGGCTCATCGCTGGGGCT	TACATTCTAGATTATTTTATTGTTTTCGATC

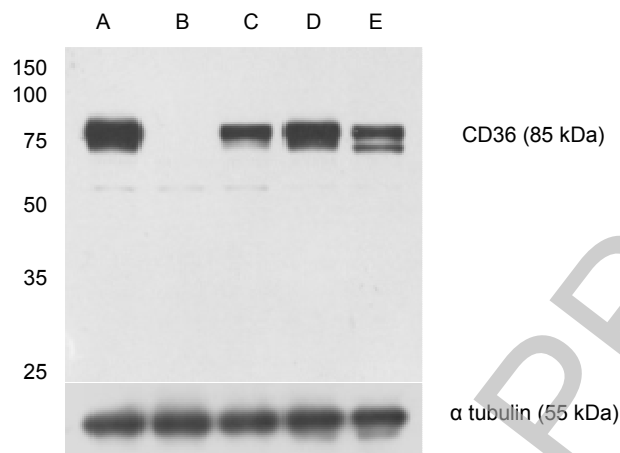
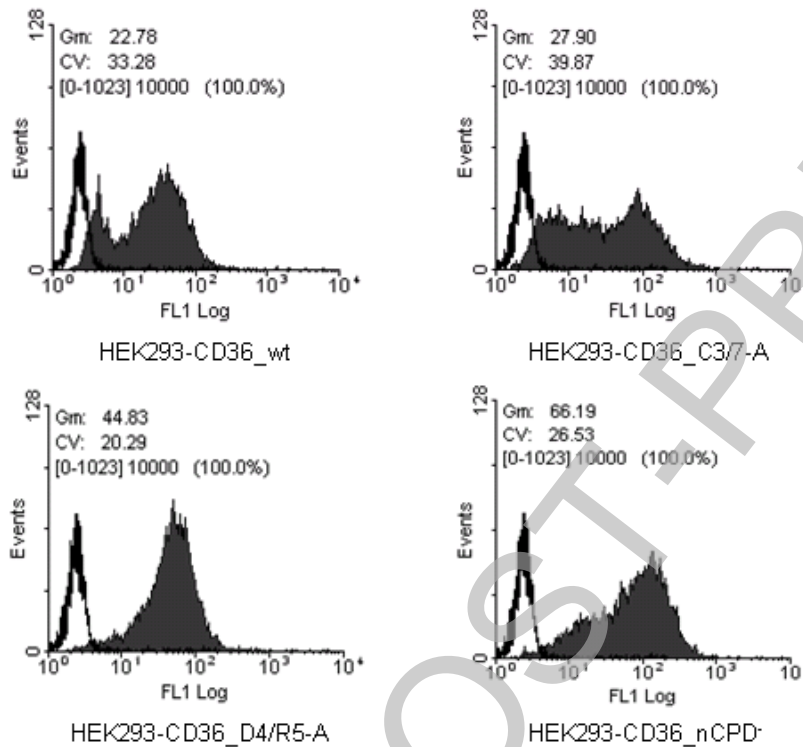


Figure 2: Detection of CD36 constructs in stably-transfected HEK293 cells by Western blotting. Triton X-100-soluble proteins obtained from stable transfectants were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was then blocked and incubated simultaneously with an anti-CD36 monoclonal antibody (10.5) and an anti- α tubulin monoclonal antibody followed by an HRP-conjugated goat anti-mouse secondary antibody. HEK293-CD36_{wt} (A), HEK293-ev (B), HEK293-CD36_{C3/7-A} (C), HEK293-CD36_{D4/R5-A} (D), and HEK293-CD36_{nCPD} (E).

(a)



(b)

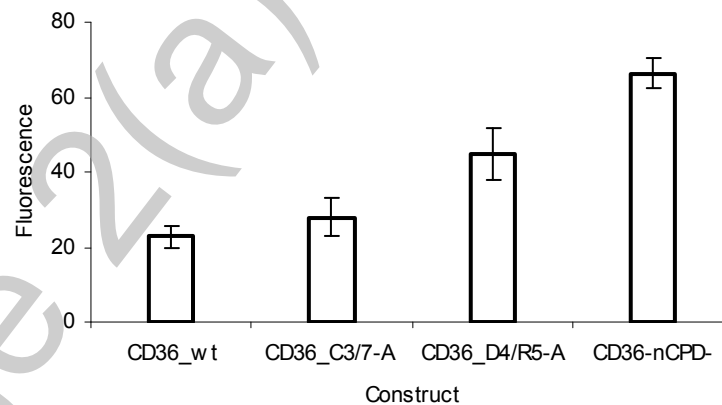


Figure 3: Analysis of CD36 construct expression in stably-transfected HEK293 cells by flow cytometry. (a) Histograms displaying qualitative expression of each construct (grey area) relative to ev-transfected cells (white over-layer). (b) Bar chart showing fluorescence (taken as an indicator of abundance) of each CD36 construct. The contribution of ev-transfected cells has been subtracted from each construct. Presented data are means \pm S.D. of 3 different experiments.

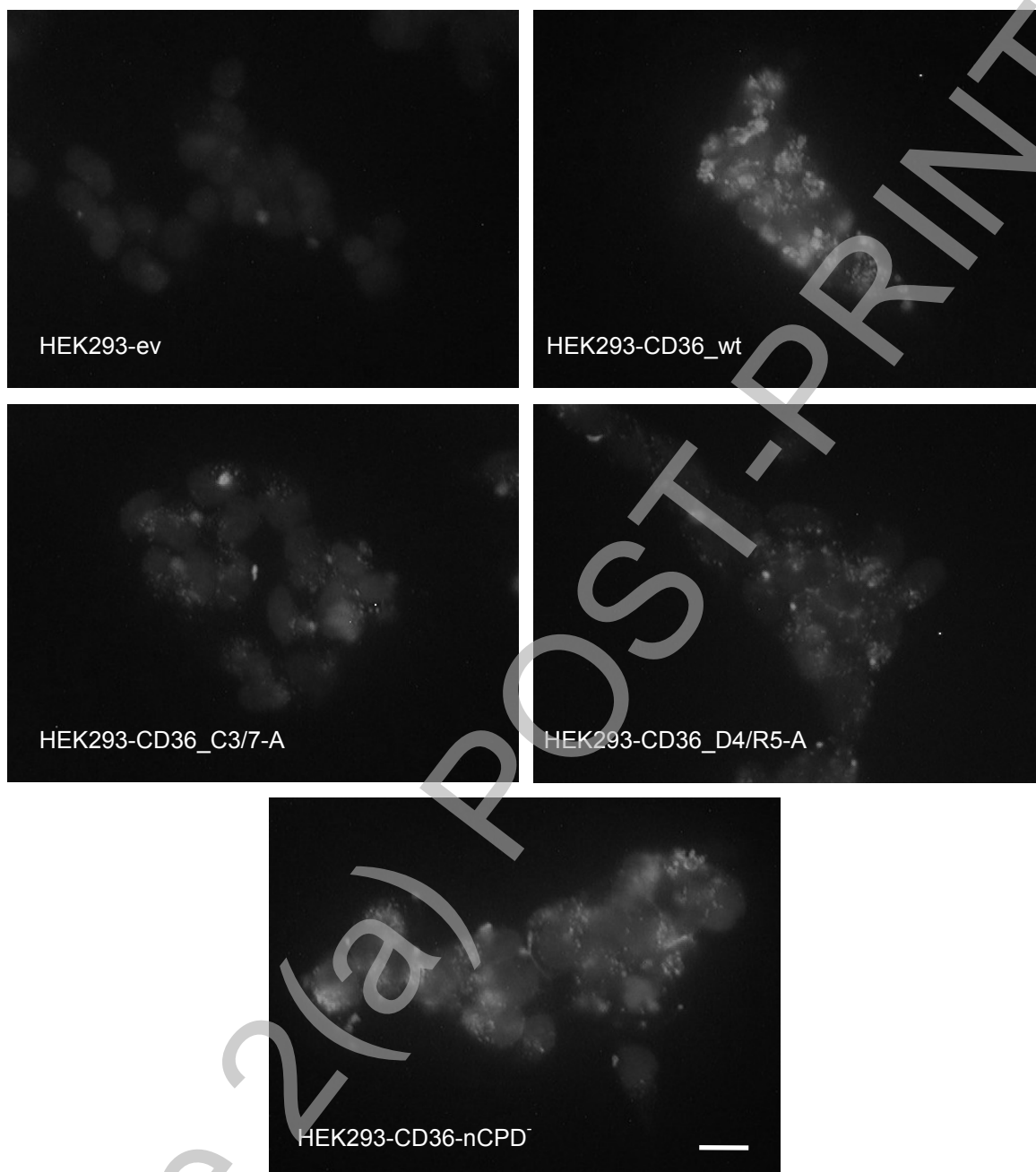


Figure 4: Visualisation of OxLDL uptake in HEK293 cells bearing different amino-cytoplasmic domain CD36 constructs. HEK293 cells stably-expressing wild-type and different CD36 amino-cytoplasmic domain constructs were grown on cover-slips in a 6-well plate (400,000 cells/well) for 24 hours before being incubated with 50 µg/ml DiI-conjugated OxLDL for 16 hours at 37°C. Empty vector (a), CD36_wt (b), CD36_C3/7-A (c), CD36_D4/R5-A (d), CD36_nCPD (e). Light coloured regions represent DiI-OxLDL. Scale bar = 10 µm.

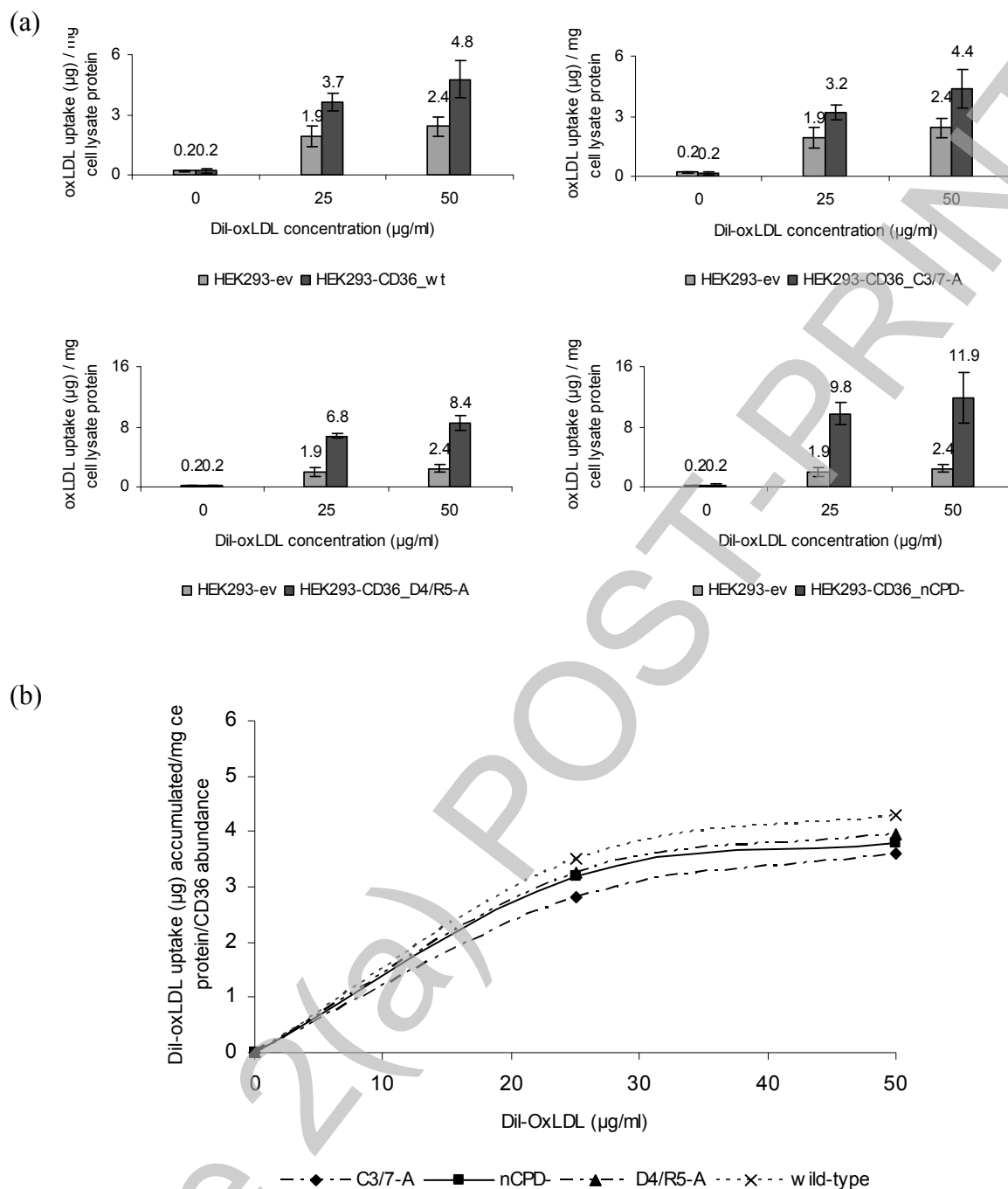


Figure 5: Accumulation of DiI-OxLDL in HEK293 cells bearing wild-type and mutant CD36 constructs.

(a) HEK293 cells stably expressing the specified CD36 constructs were incubated with 0, 25 and 50 µg/ml DiI-OxLDL for 16 hours. Cells were then lysed into 5 % Triton X-100 and DiI-OxLDL fluorescence was measured at 590 nm following excitation at 520 nm using a Molecular Devices SpectraMax Microplate Reader. Presented data are means \pm S.D. of three experiments. (b) Data from (a) after correction for CD36 abundance. Note that error bars are omitted from (b) to aid visualisation of individual data points.

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