Formation of one or more intrachain disulphide bonds is required for the intracellular processing and transport of CD36

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In monocytes/macrophages, CD36 is thought to have a role as a scavenger receptor, mediating the phagocytosis of apoptotic cells and the endocytic uptake of oxidized low-density lipoproteins and fatty acids. The proposed topology of CD36 predicts that, of ten cysteine residues, six lie in the extracellular domain, whereas four are equally distributed in the two short terminal tails flanking the N-terminal and C-terminal hydrophobic stretches. Here we investigate the formation of intrachain disulphide bonds, on the basis of the assumption that the cysteine residues present in the luminal domains are generally oxidized, whereas those in the cytosol are reduced. As revealed by gel

mobility-shift assays, disulphide bonds are present in the extracellular domain of the CD36 molecule. The formation of these bonds is required for the transport of CD36 from endoplasmic reticulum to Golgi. Furthermore reactive thiol groups are present in the CD36 sequence, which upon lysis form an intrachain extra loop as an artifact. This disulphide bond is not formed in either (1) truncated CD36 lacking the two C-terminal cysteine residues or (2) Triton X-100-insoluble wild-type CD36 molecules, suggesting that, in this fraction, the C-terminal thiol groups are modified.

INTRODUCTION

CD36 is a membrane glycoprotein expressed by erythrocyte precursors, mature monocytes, platelets, endothelial cells of the microvasculature and mammary epithelial cells [1]. In monocytes, CD36 is thought to mediate the phagocytosis of apoptotic cells [2,3] and the cytoadherence of erythrocytes infected with *Plasmodium falciparum* [4] as well as the endocytic uptake of oxidized low-density lipoproteins [5,6] and fatty acids [7]. In general, CD36 seems to have an important role as a scavenger receptor [8].

The CD36 cDNA sequence predicts a polypeptide of molecular mass 53 kDa with 10 potential N-linked glycosylation sites [9], which are processed differently in different cell types [1,10].

CD36 is a part of a family of related genes including murine CD36 [5], human CLA-1 [11], the rat proteins LIMP II [12] and FAT [7], the hamster SR-BI [13], and two *Drosophila* proteins, Croquemort [14] and emp [15].

The CD36 sequence is characterized by two hydrophobic domains: one is located at the N-terminus, whereas the other, at the C-terminus, might act as a stop-transfer sequence. Although it seems clear that the central hydrophilic domain, rich in N-glycan acceptor sites and in monoclonal antibody epitopes [16], lies extracellularly, the precise topology of this molecule remains unclear [17,18]. Of the ten cysteine residues present in the CD36 sequence, six (residues 243, 272, 311, 313, 322 and 333) are clustered in the C-terminal half of the putative extracellular domain. The remaining four cysteine residues are equally distributed at the N-terminus (positions 3 and 7) and the C-terminus (positions 464 and 466) (Figure 1A).

The conservation of the cysteine residues present in the putative extracellular domain within the CD36 protein family suggests that they might have a role in generating a common three-dimensional structure. Another conserved feature of these proteins is the presence of a hydrophobic domain at the N-terminus. However, the topology of these proteins and in

particular the role and location of the N-terminal hydrophobic domain remain controversial [1,17,18].

Because the electrophoretic mobility of native CD36 is similar under reducing and non-reducing conditions, it has been concluded that no intrachain disulphide bond(s) are formed [9,19–21]. However, this is in contrast with the notion that, whereas the cysteine residues residing in the cytosol are reduced, those located in the lumen of the endoplasmic reticulum are generally oxidized and rapidly form intra-chain or inter-chain disulphides owing to the favourable redox conditions of the exocytic pathway [22–26]. On the basis of this assumption and to clarify the secondary structure of this antigen, we have reinvestigated the formation and role of disulphide bonds in the intracellular transport of a panel of CD36 mutants.

EXPERIMENTAL

Reagents and antibodies

PMA, bicinchoninic acid solution, dithiothreitol (DTT), iodoacetamide (IAA), and *N*-ethylmaleimide (NEM) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Monobromotriethylaminobimane (MQ) was obtained from Calbiochem (San Diego, CA, U.S.A.), N-glycosidase F from Boehringer Mannheim (Mannheim, Germany) and Protein A-Sepharose CL-4B from Pharmacia (Uppsala, Sweden); enhanced chemiluminescence Western blotting detection reagents were from Amersham (Milan, Italy).

The monoclonal antibodies (mAbs) used in this study were NL07 [10], OKM5 and JCD36P (Ortho Diagnostics, Milan, Italy) and MO91 [19], all specific for CD36; the L31 anti-HLA Class I [27]; and the 3C10 anti-CD14 (American Type Culture Collection). The IgG fraction was prepared from 1207 rabbit anti-CD36 antiserum [19]. Horseradish peroxidase-conjugated rabbit anti-(mouse immunoglobulin) was obtained from Dako

Abbreviations used: DTT, dithiothreitol; IAA, iodoacetamide; mAb, monoclonal antibody; MQ, monobromotriethylaminobimane; NEM, *N*-ethylmaleimide.

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Figure 1 Structure of CD36 and mutant constructs

(A) Schematic linear representation of human CD36: cysteine residues (3, 7, 242, 272, 311, 313, 322, 333, 464, 466) are indicated as open circles, potential N-glycosylation sites as triangles and hydrophobic regions as hatched rectangles. (B) Schematic diagram of the Δ N-CD36, Δ C-CD36 and CD36-Ser^{3,7} mutant constructs. The black box indicates the cysteine residues replaced by serine residues. Abbreviation: aa, residues.

(Glostrup, Denmark). Controls were class-matched irrelevant monoclonal antibodies or normal rabbit serum.

Cell preparation and cell lines

Peripheral blood mononuclear cells were isolated from healthy donors after Ficoll–Paque gradient centrifugation in the presence of prostaglandin E₂ to minimize platelet activation and adhesion to monocytes. Cells at the interface were removed and washed twice in PBS. After 1 h of incubation at 37 °C in 60 mm Petri dishes, non-adherent cells were removed by washing the plates several times in PBS. More than 90 % of the adherent cells were monocytes as revealed by reactivity with anti-CD14 mAb. In selected experiments, washed monocytes were incubated (45 min at 37 °C in PBS) in the presence of either membrane-permeable (10 mM IAA or 5 mM NEM) [28] or membrane-impermeable (5 mM MQ) [29,30] alkylating agents and washed three times with PBS before lysis.

The premonocytic cell line U937 was maintained in culture as previously described [10]. U937 cells were plated at 10⁶/ml in Petri dishes and treated with PMA (40 nM) for 14 h.

SDS/PAGE and Western blotting

Cells (3 × 10⁶) were washed twice with Tris-buffered saline [TBS; 10 mM Tris/HCl (pH 7.4)/150 mM NaCl], resuspended at 10⁶ cells/ml and lysed with Triton X-100 [final concentration 1 % (v/v)] for 30 min at 0 °C in the presence of protease inhibitors and with or without different alkylating agents (10 mM IAA, 5 mM NEM or 5 mM MQ). After centrifugation for 15 min at 12000 g, the Triton X-100-soluble lysates were removed and the fraction insoluble in 1 % Triton X-100, hereafter referred to as ‘Triton X-100-insoluble’, was extracted in the presence of protease inhibitors with TBS containing 1 % (w/v) SDS (30 min at 20 °C) and sonicated to fragmentate DNA (three treatments at 200 W for 20 s, on ice). Both Triton X-100-soluble and Triton X-100-insoluble fractions were resolved by SDS/PAGE on vertical slab gels containing 7.5 % or 10 % (w/v) polyacrylamide. In

some experiments cells were incubated (30 min at 4 °C) before lysis with either NL07 anti-CD36 or L31 anti-HLA Class I mAb and then washed twice with TBS. In each experiment, samples were normalized so as to contain the same amount of total protein as determined by the bicinchoninic acid assay (Sigma), reduced by treatment with 2-mercaptoethanol and successively alkylated by treatment with IAA. Proteins were electro-transferred (2 h at 60 V) to nitrocellulose filters. The latter were preincubated for 1 h at 20 °C with 5 % (w/v) BSA in TBS and incubated with purified mAbs specific for CD36 (Mo91; final dilution 1:400), HLA CI I (L31; final dilution 1:1000). After 1 h the blots were washed with TBS containing 0.1 % Tween-20, incubated with horseradish peroxidase-conjugated rabbit anti-(mouse immunoglobulin) (1:1000) for 1 h and processed for enhanced chemiluminescence in accordance with the supplier's instructions.

The molecular mass standards included lysozyme (14 kDa), trypsin inhibitor (21 kDa), carbonic anhydrase (30 kDa), ovalbumin (46 kDa), BSA (69 kDa), phosphorylase b (97 kDa), β -galactosidase (116 kDa) and myosin (200 kDa), and were revealed by Ponceau Red staining.

Endogenous labelling

For pulse–chase experiments cells were preincubated for 15 min in methionine/cysteine-free medium supplemented with 2 % (v/v) dialysed FCS and 1 mM glutamine and labelled at 2 × 10⁷/ml for 25 min with 18.5 MBq/ml Tran³⁵S-label (ICN Radiochemicals, Milan, Italy). Pulse-labelled cells were then washed and chased in regular culture medium with or without 5 mM DTT for various periods before lysis and immunoprecipitation as indicated in the figure legends.

Immunoprecipitation

After a preclearing step (2 h at 4 °C) with normal rabbit serum adsorbed on Protein A–Sepharose, cell lysates were immunoprecipitated with Protein A–Sepharose beads coated with the

relevant antibodies (1 h at 4 °C). After washing in 0.1 % Triton X-100 TBS, the immunocomplexes were eluted by boiling for 5 min in sample buffer and resolved by SDS/PAGE. Gels were fixed and processed with Amplify (Amersham, Milan, Italy) before analysis with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Deglycosylation of glycoproteins

Approx. 20 µg of total cellular proteins from Triton X-100-soluble or Triton X-100-insoluble fractions were boiled for 2 min in the presence of 0.2 % SDS, diluted in 100 µl of the appropriate buffers as specified by the manufacturers, and incubated with or without 1 unit of N-glycosidase F for 18–24 h at 37 °C.

Construction and expression of the ΔN- and ΔC-truncated CD36 and the CD36-Ser^{3,7} mutants

The ΔC-truncated CD36 construct (ΔC-CD36) lacking residues 437–471 is a deletion of the C-terminal hydrophobic domain and cytoplasmic tail. ΔC-CD36 was generated by site-directed mutagenesis *in vitro* (QuikChange Site Directed Mutagenesis Kit; Stratagene, Basel, Switzerland), with oligonucleotide primers containing two stop codons, each complementary to opposite strands of the vector (5'-GT CAA GTA ACT GGA TAA TAG AAG CTT CTT GGC CTG ATA G-3'; 5'-C TAT CAG GCC AAG AAG CTT CTA TTA TCC AGT TAC TTG AC-3'). The ΔN-truncated CD36 construct (ΔN-CD36), lacking residues 1–29, does not contain the hydrophobic domain and the short N-terminal tail. In this construct, the cleavable leader sequence derived from the Sp6 V_H immunoglobulin gene has been inserted so as to generate a polypeptide beginning at residue 30. The fragment of interest was subcloned from pUC18 plasmid containing CD36 cDNA (made available by Dr. R. Lipsky, American Red Cross, Rockville, MD, U.S.A.) [31]; the Sp6-cleavable leader sequence was added by using a PCR-based gene assembly technique [32]. For both constructs, the amplified products of the correct sizes were inserted into the Bluescript M13 plasmid (Stratagene) and sequenced. The Bluescript CD36 inserts were then subcloned into pSRI/neo plasmid (ΔN-CD36-pSRI/neo; ΔC-CD36-pSRI/neo).

Using PCR mutagenesis [33] we generated a CD36 mutated in the cysteine residues in positions 3 and 7 (CD36-Ser^{3,7}). The specific upstream primer (5'-ggcagaattc ATG GGC TCT GAC CGG AAC TCT GGG C-3'), used to amplify a fragment from pUC18 plasmid containing CD36 cDNA, replaced both cysteine residues with serine residues introducing also an *EcoRI* restriction site at the 5' of the mutated CD36. The downstream primer used (5'-C⁷²⁸C AAC TGT AGT AGT AAC AGG GTA CGG AAC-3') included in the generated fragment the *BamHI* restriction site present at position 681 in the native CD36. The *EcoRI*–*BamHI*-digested fragment was gel-purified, subcloned into pBluescript II and resequenced. The same fragment was ligated to the 1120 bp fragment generated by *EcoRI*–*BamHI* digestion of pUC18-CD36wt cDNA and the purified product of the correct size and orientation was subcloned into a pSRI/neo plasmid (CD36-Ser^{3,7}-pSRI/neo).

Samples (9 µg) of native CD36-pSRI/neo, mutated CD36-Ser^{3,7}-pSRI/neo, ΔN-CD36-pSRI/neo, ΔC-CD36-pSRI/neo or pSRI/neo alone were used to transfect COS-7 cells transiently with cationic polymer polyethylenimine (Sigma) as described [34]. The cells were cultured for 48 h in Dulbecco's modified Eagle's medium supplemented with 5 % (v/v) FCS and used for biochemical analyses. Surface expression of transfected antigens was monitored by flow cytometry analyses after staining with anti-CD36 specific mAb as described [35].

RESULTS

CD36 contains disulphide bond/s

The presence of intrachain disulphide bonds in a protein can be revealed by differing electrophoretic mobilities under reducing and non-reducing conditions ([25], and references therein). In glycoproteins, however, subtle differences might be obscured by the heterogeneity in glycan processing. Indeed, the existence of intrachain disulphides in CD36 becomes evident only on complete deglycosylation with N-glycosidase F (Figure 2; compare lanes 3 and 4). The difference in electrophoretic mobility between reduced and non-reduced samples is not appreciable if glycans are not removed (Figure 2, lanes 1 and 2). This probably explains why intrachain disulphide bonds have not been detected in CD36 by others [19–21].

Disulphide bond formation is required for processing the 74 kDa CD36 intracellular precursor

As previously observed [35], CD36 is synthesized as an endoglycosidase H-sensitive 74 kDa precursor, which is converted into endoglycosidase H-resistant species of lower mobility (Figure 3, lanes 1–4) in the Golgi. To investigate the role of disulphide bonds in the intracellular trafficking of CD36, we exploited DTT. This strong, divalent reducing agent effectively prevents disulphide bond formation in newly synthesized proteins, and blocks the exit from the endoplasmic reticulum of those molecules in which folding and/or assembly require thiol oxidation [24,25,36]. When DTT is added during the chase, the processing of CD36 is inhibited and the precursor is slowly degraded (Figure 3, lanes 5–7). At the end of the pulse (Figure 3, lane 1) an intense band of 33 kDa is precipitated by anti-CD36 antibodies. As previously shown, this molecule (gp33) disappears rapidly during the chase (Figure 3, lanes 1–4), and even more so in the presence of DTT (Figure 3, lanes 5–7). The preferential precipitation of gp33 from cells in which the transport of CD36 was particularly slow suggested that it might be a CD36-associated protein [35]. However, because our antibody also precipitates gp33 from non-transfected COS-7 cells (P. Guarini and M. Alessio, unpublished work), the nature of this rapidly degraded band remains to be determined. The presence of DTT also prevents the co-precipitation of gp33. The effects of DTT are in part reversible: when cells exposed to DTT for 30 min are

Figure 2 Intrachain disulphide bonds in CD36

Monocytes were lysed in the presence of 10 mM IAA. Aliquots from the lysates were treated with (+) or without (–) N-glycosidase F (N-GlyF) and resolved by SDS/PAGE [10 % (w/v) gel] under reducing (+ 2-ME) or non-reducing (– 2-ME) conditions with 2-mercaptoethanol. Gels were transferred to a nitrocellulose membrane decorated with anti-CD36 mAb (Mo91). Lines at the right indicate the observed electrophoretic mobility shift. The positions of molecular mass markers (in kDa) are indicated at the left.

Figure 3 Effects of DTT on the maturation of CD36

(A) Scheme of the experiment. U937 cells treated for 12 h with PMA to increase CD36 synthesis were pulse-labelled for 20 min with [35 S]methionine/cysteine, washed and chased for the indicated periods in the presence (lane 5) or absence (lanes 2, 3 and 4) of 5 mM DTT. After a chase for 30 min in the presence of DTT, aliquots of cells were washed and then chased further with (lanes 6 and 7) or without (lanes 8 and 9) the reducing agent before lysis. Lysates were immunoprecipitated with anti-CD36 (1207), and resolved by SDS/PAGE (B). The arrow points to the co-immunoprecipitated gp33, the line indicates the mature CD36 protein, and the arrowhead the CD36 precursor. The positions of molecular mass markers (in kDa) are indicated at the left.

further chased in the absence of the reducing agent, the precursor matures into the 95 kDa endoglycosidase H-resistant form (Figure 3, lanes 8 and 9). It should be noted that, in cells exposed to DTT, the electrophoretic mobility of the CD36 precursor is slightly greater than in controls (Figure 3, compare lanes 3, 6 and 8). At present the reasons for this difference are unclear.

New intrachain disulphide bonds are formed when cells are lysed in the absence of alkylating agents

When monocytes are lysed in the absence of alkylating agents, a new CD36 species is detected in the deglycosylated and non-reduced sample (Figure 4, see arrow on lane 2). This form, which is absent when alkylating agents are present at the moment of lysis (Figure 4, lanes 4 and 6), probably originates from the post-lysis disulphide bonding of two free reactive thiols present in CD36. We interpret this mobility shift as an intrachain disulphide bond formed in a subset of CD36 molecules on lysis. A less likely explanation is that reactive cysteine residues of CD36 form intermolecular bonds that confer a greater electrophoretic mobility.

Which are the cysteine residues responsible for the post-lysis formation of the extra disulphide bond in CD36? In general, cytosolic proteins contain reduced cysteine residues, whereas oxidized cysteine residues predominate in secretory molecules [22,26,37]. Hence it was likely that cysteine residues present in the cytosolic domains are involved in the formation of the extra

Figure 4 Post-lysis formation of intrachain disulphide bond(s) in CD36

Monocytes were lysed either in the absence of alkylating agents (lanes 1 and 2) or after 45 min treatment with membrane-permeable (NEM) or membrane-impermeable (MQ) alkylating agents (lanes 7–10). Lysis of untreated monocytes was also performed in the presence of either 5 mM NEM or 5 mM MQ (lanes 3–6). Aliquots from the lysates were treated with N-glycosidase F and resolved by SDS/PAGE [10% (w/v) gel] under reducing (+ 2-ME) or non-reducing (– 2-ME) conditions with 2-mercaptoethanol. Gels were transferred to a nitrocellulose membrane decorated with anti-CD36 (Mo91). Lines at the right indicate the electrophoretic mobility shift observable under reducing conditions, and the arrows indicate the extra bands formed after lysis. The positions of molecular mass markers (in kDa) are indicated at the left.

loop. To verify this possibility, we made use of two alkylating agents, MQ and NEM, that differ in their membrane permeabilities, only the latter being able to cross a lipid bilayer in intact cells [28]. As shown in Figure 4, the formation of the extra loop is blocked when monocytes are treated before lysis with NEM (lane 10), but not with the membrane-impermeable MQ (lane 8), suggesting that the reactive cysteine residues are inaccessible. When present during the lysis, both alkylating agents block the formation of this disulphide bond artifact (Figure 4, lanes 4 and 6).

The absence of homo-oligomers or hetero-oligomers suggests that the formation of intrachain disulphide bonds is favoured, probably because of the proximity of the two reactive thiols.

Presence of a Triton X-100-insoluble fraction of CD36 in monocytes that does not form the post-lysis intrachain disulphide bonds

In endothelial cells, part of CD36 is not soluble in non-ionic detergents such as Triton X-100. In addition, in unstimulated monocytes, which contain primarily the mature form of CD36, from one-third to one-half of the molecules accumulate in the Triton X-100-insoluble pellets (Figure 5, compare lanes 1 and 5). Although similar in electrophoretic mobility, Triton X-100-soluble and Triton X-100-insoluble CD36 molecules display a remarkable difference in that only the former can form the post-lysis intrachain disulphide bond on lysis in the absence of alkylating agents. As evident in Figure 5 (compare lanes 4 and 8), the extra loop is not formed as efficiently by the Triton X-100-insoluble CD36 molecules.

Expression of the Triton X-100-insoluble fraction of CD36 on the plasma membrane

In view of the differences between Triton X-100-soluble and Triton X-100-insoluble CD36 with respect to disulphide bond formation, we decided to determine whether both fractions were expressed on the plasma membrane and are thus equally

Figure 5 Decreased formation of the post-lysis intrachain disulphide bond(s) in Triton X-100-insoluble CD36

Monocytes were lysed with 1% Triton X-100 and the Triton X-100-soluble (sol; lanes 1–4) and Triton X-100-insoluble (insol; lanes 5–8) fractions were separated by centrifugation. After SDS solubilization of the Triton X-100-insoluble fraction, aliquots from the lysates were treated with (+) or without (–) N-glycosidase F (N-GlyF) and resolved by SDS/PAGE [10% (w/v) gel] under reducing (+2-ME) or non-reducing (–2-ME) conditions with 2-mercaptoethanol. Gels were transferred to a nitrocellulose membrane decorated with anti-CD36 (Mo91). The arrow indicates the extra band formed after lysis. The positions of molecular mass markers (in kDa) are indicated at the left.

accessible to exogenously added antibodies. To investigate this issue, live U937 cells were incubated with anti-CD36 antibodies (NL07, an IgM mAb) or with anti-HLA Class I (L31, an IgG mAb) before lysis with Triton X-100 and separation of the Triton X-100-soluble and Triton X-100-insoluble fractions (see the Experimental section for further details).

In this experiment, about half of the mature CD36 molecules accumulated in the Triton X-100-insoluble fraction (Figure 6A, lanes 3 and 4). HLA Class I molecules were present exclusively in the Triton X-100-soluble fraction, confirming that insolubility is not the result of antibody-induced cross-linking (Figure 6A, lane 1). Accordingly, the exogenous anti-HLA antibodies (revealed by decorating the blot with anti- γ antibodies) are found exclusively in the Triton X-100-soluble fraction (compare lanes 1 and 2 of Figure 6B). In contrast, anti- μ antibodies decorate the Triton X-100-insoluble fractions slightly more intensively than the Triton X-100-soluble fraction (Figure 6B, lanes 3 and 4), indicating that both the Triton X-100-soluble and Triton X-100-insoluble fractions of CD36 are expressed on the cell surface.

As previously reported [35], only the mature form of CD36 (more than 95 kDa) is detectable in unstimulated U937. Treatment with PMA induces the synthesis of the 74 kDa CD36 precursor. This immature form is found entirely in the Triton X-100-soluble pool (Figure 6C), indicating that the acquisition of Triton X-100 insolubility is a late event during the biogenesis of CD36.

Replacing cysteine residues 3 and 7 by serine residues prevents the partitioning of CD36 in the Triton X-100-insoluble fraction without affecting surface expression and post-lysis intrachain disulphide bond formation

According to the hairpin topology proposed by Tao et al. [18] for CD36, with two transmembrane domains at the N-terminus and the C-terminus, the reducing environment of the cytosol could physiologically maintain the four terminal cysteine residues in the reduced state, ready to form covalent bonds on oxidation after lysis. The most likely interpretation of the formation of the

Figure 6 Triton X-100-insoluble CD36 is accessible to exogenous antibodies

(A) Partitioning of CD36 and HLA Class I antigens in Triton X-100-soluble and Triton X-100-insoluble fractions. U937 cells were lysed with Triton X-100; Triton X-100-soluble (sol) and Triton X-100-insoluble (insol) fractions were separated by centrifugation. Lysates were resolved by SDS/PAGE [10% (w/v) gel] under reducing conditions. Gels were transferred to nitrocellulose membranes decorated with either anti-CD36 mAb (CD36) or anti-HLA Class I mAb (HLA CII). The positions of molecular mass markers (in kDa) are indicated at the left. (B) Partitioning of antibodies that have reacted with surface antigens in Triton X-100-soluble or Triton X-100-insoluble fractions. U937 cells were incubated with an IgM mAb anti-CD36 (α -CD36) or with an IgG mAb anti-HLA Class I (α -HLA CII) before lysis with Triton X-100 and separation of the Triton X-100-soluble (sol) and Triton X-100-insoluble (insol) fractions as in (A). Lysates were resolved by SDS/PAGE [10% (w/v) gel] under reducing conditions. Gels were transferred to nitrocellulose membranes decorated either with anti- γ antibodies (α -IgG) or anti- μ antibodies (α -IgM). (C) The 74 kDa CD36 precursor does not become Triton X-100-insoluble. Untreated (–) U937 cells, or cells treated (+) for 12 h with 40 nM PMA, were lysed with Triton X-100; Triton X-100-soluble (sol) and Triton X-100-insoluble (insol) fractions were separated. Lysates were resolved by SDS/PAGE [10% (w/v) gel] under reducing conditions. Gels were transferred to nitrocellulose membranes decorated with an anti-CD36 mAb. The bracket at the right indicates the mature form of CD36; the arrow indicates the 74 kDa CD36 precursor.

extra loop is the bridging of one (or both) cysteine residues located in the two N- and C-termini. Should this be the case, replacing both cysteine residues present at one of the protein extremities should block the extra loop formation. To address this point we generated a mutated CD36 with Cys-3 and Cys-7 replaced by serine residues.

Cytofluorimetric analysis revealed that both the wild-type CD36 and the CD36-Ser^{3,7} mutant are expressed on the surface

Figure 7 Cysteine residues 3 and 7 have a role in the solubility of CD36 in Triton X-100 but do not affect CD36 surface expression and post-lysis intrachain disulphide bond formation

(A) Flow cytometry analysis of the surface expressions of native CD36 and CD36-Ser^{3,7} mutant. Indirect immunofluorescence tests were performed on COS-7 cells transiently transfected with plasmids containing either native (CD36wt) or mutated CD36 (CD36-Ser^{3,7}) with the JCD36P anti-CD36 mAb. The detection antibody was FITC-conjugated goat anti-(mouse immunoglobulin). The shaded peak indicates background fluorescence on control cells transfected with plasmid alone. The ordinate shows cell numbers; the abscissa fluorescence intensity. (B) Partitioning of CD36 mutated at cysteine residues 3 and 7 between Triton X-100-soluble and Triton X-100-insoluble fractions. COS-7 cell transfectants expressing native CD36 (wt) or CD36 mutated at cysteine residues 3 and 7 (CD36-Ser^{3,7}) were lysed with 1% Triton X-100; the Triton X-100-soluble (sol) and Triton X-100-insoluble (insol) fractions were separated by centrifugation. After solubilization of the Triton X-100-insoluble fraction with SDS, aliquots from the lysates were resolved by SDS/PAGE [10% (w/v) gel] under reducing conditions. Gels were transferred to a nitrocellulose membrane decorated with anti-CD36 mAb (Mo91). The positions of molecular mass markers (in kDa) are indicated at the left. (C) Formation of a post-lysis intrachain disulphide bond in CD36 mutated at cysteine residues 3 and 7. COS-7 cell transfectants expressing mutated CD36-Ser^{3,7} were lysed in the presence (+) or absence (–) of the alkylating agent IAA. Aliquots from the lysates were treated with N-glycosidase F and resolved by SDS/PAGE [10% (w/v) gel] under reducing (+2-ME) or non-reducing (–2-ME) conditions with 2-mercaptoethanol, and processed as indicated in (B). The arrow indicates the extra band formed by post-lysis oxidation.

of COS-7 transfectants. A range of 20–30% of transiently transfected cells expressed native or mutated CD36 (Figure 7A).

In transfected COS-7 cells most mature CD36 molecules partition in the Triton X-100-insoluble fraction (Figure 7B, lanes 1 and 2). Interestingly, replacing cysteine residues 3 and 7 by serine residues modified the physicochemical properties of the mature CD36, which became mostly Triton X-100-soluble (Figure 7B, lanes 3 and 4).

However, the fraction of Triton X-100-soluble mutated CD36-Ser^{3,7} that formed the post-lysis intrachain disulphide bond when lysis occurred in the absence of an alkylating agent (Figure 7C, lane 2) is similar to the wild type, indicating that cysteine residues

Figure 8 C-terminal cysteine residues are involved in the post-lysis intrachain disulphide bond formation

COS-7 cells transfected with native CD36, or N-terminally or C-terminally truncated CD36 (ΔN-CD36 and ΔC-CD36 respectively), were lysed either in the absence (lanes 1, 2, 5, 6, 9 and 10) or in the presence (lanes 3, 4, 7, 8, 11 and 12) of the alkylating agent IAA at 5 mM. Aliquots from the lysates were treated with N-glycosidase F and resolved by SDS/PAGE [10% (w/v) gel] under reducing (+2-ME) or non-reducing (–2-ME) conditions with 2-mercaptoethanol. Gels were transferred to a nitrocellulose membrane decorated with anti-CD36 (Mo91). The arrows indicate the extra band formed by post-lysis oxidation. The positions of molecular mass markers (in kDa) are indicated at the left.

at the N-terminus of CD36 are not involved in the formation of the extra disulphide bond.

C-terminal cysteine residues are involved in the formation of the intrachain disulphide bond after lysis

To understand which cysteine residues are involved in the formation of the extra loop we generated N-terminally and C-terminally truncated CD36 molecules (ΔN-CD36 and ΔC-CD36 respectively). Confirming that cysteine residues 3 and 7 are not involved, the ΔN-CD36 mutant formed the post-lysis intrachain disulphide bond, similarly to wild-type molecules (Figure 8, lanes 2 and 6). In contrast, the ΔC-CD36 mutant, lacking both the C-terminal cysteine residues, did not show mobility shifts, suggesting that either one or both of Cys-464 and Cys-466 are involved in the post-lysis intrachain disulphide bond (Figure 8, lane 10).

DISCUSSION

Most secretory and membrane proteins contain disulphide bonds that are often crucial for their function and stability as well as for their maturation and intracellular transport. The disulphide bonds are generally formed in the endoplasmic reticulum, where a suitable redox potential [23] and a vast array of oxidoreductases and chaperones create an optimal environment for folding, assembly and oligomerization [38].

In addition the CD36 antigen forms intrachain disulphide bonds that appear to be essential for its intracellular transport and structural biogenesis. It is probably the abundant and heterogeneous glycosylation that, in previous studies [9,19–21], prevented the detection of these disulphide bonds by the standard mobility assays in reducing compared with non-reducing conditions. Indeed, unless CD36 is deglycosylated, the subtle differences in electrophoretic migration are not appreciable.

In general, the more distant the cysteine residues that form an intrachain bond, the bigger is the mobility difference between reduced and non-reduced samples. Because many of the cysteine residues located in the putative extracellular domain are rather close to each other, it is not possible at present to determine the number and the order of disulphide bonds in native CD36 molecules.

The effect of DTT (Figure 3) is a strong indication that CD36 contains disulphide bonds essential for its structural maturation. In the presence of DTT, newly made CD36 is retained and slowly degraded. On removal of the reducing agent, the CD36 precursor can mature rapidly into a transport-competent form that acquires the modifications typical of the late Golgi stacks and is eventually expressed on the cell surface.

Interestingly, when live U937 cells are exposed to DTT, the intrachain disulphide bonds present in surface CD36 molecules are reduced; reactivity with Mo91, a monoclonal antibody specific for denatured CD36 [39], ensues (P. Gruarin, R. Sitia and M. Alessio, unpublished work). These observations raise the possibility that the function of CD36 is regulated by the extracellular redox state.

At present it is not clear why the gel mobility of the CD36 precursor is slightly greater in the presence of reducing agents. Further experiments are necessary to determine whether some of the cysteine residues (and, if so, which) interact with other molecules in a DTT-sensitive way.

That not all of the cysteine residues present in the CD36 sequence are involved in disulphide bonds is suggested by the changes in electrophoretic mobility that are observed in deglycosylated CD36 molecules when reactive thiols are not blocked by alkylation during cell lysis.

This post-lysis modification involves cysteine residues that are not blocked when impermeant alkylating agents are administered before cell lysis. Because the diffusibility of NEM and MQ in solution should be similar, the most likely explanation is that at least one of the cysteine residues involved is located in the cytosolic portion(s) of CD36. In the hairpin model of CD36 suggested by the experiments of Tao et al. [18], the four terminal cysteine residues lie in the cytosol. The reducing environment of this compartment could maintain these residues physiologically in the thiol state, ready to form covalent bonds on oxidation after lysis. It is noteworthy that homopolymers or heteropolymers are not detected when cells are lysed in the absence of alkylating agents. This suggests that the reactive thiols might be in close proximity, thereby favouring the formation of the intrachain bond(s), although the remarkable mobility shift caused by this bond would be more likely to reflect a link between cysteine residues distant from one another within the polypeptide. However, the most likely interpretation, i.e. the bridging of one or both cysteine residues located at the two N- and C-termini, is untenable. Indeed, the mobility shift is also observed in the two mutants lacking cysteine residues 3 and 7 (Δ N-CD36 and CD36-Ser^{3,7}). In contrast, the involvement of one or both C-terminal cysteine residues (464 and 466) is indicated by the failure of mobility shift in Δ C-CD36. The proximity of the two C-terminal cysteine residues makes it unlikely that the electrophoretic mobility shift observed is due to the formation of a covalent bond between them. A possible interpretation is that the extra loop is formed between one of the C-terminal cysteine residues and one in the extracellular domain. In this case, the cysteine residue in the extracellular domain should be (in its native configuration) inaccessible to MQ but ready to form a disulphide on lysis. Detergents could then distort the molecule sufficiently to expose the thiol without losing reactivity with NL07 mAb and other antibodies recognizing conformational epitopes.

As with endothelial cells [40] and platelets [41], a fraction of CD36 becomes Triton X-100-insoluble also in monocytes and monocytic cell lines (Figures 5 and 6). Triton X-100 insolubility is thought to result from the incorporation of membrane proteins into large complexes that contain sphingolipids and cholesterol, and might include cytoplasmic signalling molecules such as Src family tyrosine kinases and GTP-binding proteins [42–46]. The

absence of Triton X-100-insoluble 74 kDa CD36 precursors suggests that the critical modifications are acquired in late compartments of the exocytic pathway, possibly in or beyond the Golgi where glycolipids are synthesized [46]. Indeed, a large fraction of Triton X-100-insoluble CD36 is accessible to antibodies (Figure 6). The expression of a detergent-specific conformational isoform or heterodimeric complex of CD36 has been reported [47].

The failure of the Triton X-100-insoluble CD36 to undergo mobility shift and the observation that Cys-3 and Cys-7 have a role in the solubility of CD36 in Triton X-100 are in line with the observation of Tao et al. [18] showing an S-ester linkage of palmitoyl groups to the N-terminal and C-terminal CD36 cysteine residues. It is possible that the Triton X-100 insolubility of CD36 involves the acylation (palmitoylation or myristoylation) of one or both the cytosolic tails. Indeed, in platelets the Triton X-100-insoluble fraction of CD36 has been reported to be palmitoylated [41]. This modification would prevent the formation of disulphide bonds.

Another possibility is that the cytosolic cysteine residues mediate the association with signalling molecules, similarly to p56lck with CD4 or CD8. In platelets, signal transduction through CD36 [1,48–50] is thought to involve an association with the pp60c-Src-related tyrosine kinases Fyn, Lyn and Yes [51], possibly mediated by the sequence CXCX₅K present in the C-terminal tail of CD36 [52]. The kinase proteins, which are also enriched in the Triton X-100-insoluble fraction [45], could prevent intrachain disulphide formation or other modifications in CD36 by masking the critical thiol groups or decreasing their accessibility.

In conclusion, our results indicate that disulphide bonds are formed in the extracellular domain of CD36 that are essential for the maturation and transport of the protein. Moreover, the two N-terminal intracellular cysteine residues are important for the acquisition of Triton X-100 insolubility, but not all surface-expressed CD36 molecules are Triton X-100-insoluble. Whether this is related to a modulatable functional role of CD36 is under investigation.

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