Identification of an immunodominant functional domain on human CD36 antigen using human–mouse chimaeric proteins and homologue-replacement mutagenesis

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The human CD36 antigen is a multifunctional membrane glycoprotein that acts as a receptor for thrombospondin, malaria-infected erythrocytes and oxidized low-density lipoprotein, as well as being implicated in the recognition of apoptotic neutrophils by macrophages. OKM5 and other anti-CD36 monoclonal antibodies have been shown to inhibit these CD36 adhesive functions, suggesting that the monoclonal-antibody epitopes and the domains that mediate these events are closely related. Analysis of a series of chimaeric exchanges between human and mouse CD36 shows that six anti-CD36 monoclonal antibodies (OKM5, FA6-152, L103, 5F1, SMΦ and 10/5) recognize epitopes within the domain comprising amino acids 155–183. A seventh monoclonal antibody (13/10) binds to another domain that spans amino acids 30–76. Homologue-replacement mutagenesis performed within the human 155–183 immunodominant sequence identifies key residues for the binding of three functional monoclonal antibodies (OKM5, FA6-152 and L103). The fact that antibodies directed against the 155–183 domain can inhibit adhesion suggests that this domain is directly involved in CD36–ligand binding.

INTRODUCTION

Glycoprotein CD36, also known as GPIIIb or GPIV, is a multifunctional adhesive receptor expressed by platelets, monocytes, endothelial cells and various tumour-cell lines. This 88 kDa glycoprotein acts as a receptor for thrombospondin [1], collagen type I [2], *Plasmodium falciparum*-infected erythrocytes [3] and oxidized low-density lipoprotein [4]. CD36 has also been shown to be involved as a signal-transmission molecule in platelets [5,6] and monocytes [7]. Moreover, CD36 in tandem with $\alpha_v\beta_3$, a member of the integrin family, is implicated in the recognition and phagocytosis of apoptotic neutrophils by macrophages [8].

Human CD36 cDNA has been isolated from placenta by expression cloning [3] and from platelets by mRNA amplification [9]. Two recently cloned glycoproteins, human CLA-1 [10] and rat LIMPII [11], are structuraly related to CD36, suggesting that these proteins belong to a new gene family. Moreover, murine [4] and rat [12] homologues of human CD36 have been isolated. At present, little is known on the structural/functional domains that modulate the binding of ligands to CD36 or that define antigenic sites on this receptor. Recently, the binding site of a functional anti-CD36 monoclonal antibody (mAb), OKM5, was mapped to amino acids (aa) 139–155 [13]. However, a synthetic peptide derived from aa 139–155 did not inhibit the binding of OKM5 to CD36-transfected COS cells [14]. In the present study, we report the epitope mapping of the functionally important anti-CD36 mAb OKM5 and of a panel of six other anti-CD36 mAbs using human–mouse chimaeric proteins and homologue-replacement mutagenesis. The potential functional properties of the identified sequences are discussed.

MATERIALS AND METHODS

Materials

The M13 mutagenesis kit was obtained from Bio-Rad (Richmond, CA, U.S.A.). Oligonucleotides were from Genosys (The Woodlands, TX, U.S.A.). Restriction endonucleases were from New England Biolabs (Beverly, MA, U.S.A.) or Gibco–BRL (Gaithesburg, MD, U.S.A.). LipofectAmine reagent was from Gibco–BRL. The COS-7 cell line was obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, Wiltshire, U.K.). The clones expressing human and mouse CD36 were generously given by Dr. Brian Seed [3] and Dr. Gerda Endemann [4] respectively.

CD36 constructs

Human and murine CD36 cDNAs were both cloned into the *Hin*dIII and *Xba*I sites of M13mp19. To facilitate the generation of chimaeric CD36 proteins, restriction-endonuclease sites were introduced into the human and mouse cDNAs without altering the coding sequence. The mutated cDNAs were then subcloned into the pCDM8 expression vector, digested with the appropriate restriction endonucleases to allow exchange of sequences between human and mouse cDNAs and the ligation products transformed into *Escherichia coli* MC1061p3. When homologue-replacement mutagenesis was used, aa substitutions were introduced into the human cDNA, again by oligonucleotide-directed mutagenesis. In most cases, codon degeneracy was exploited to introduce a novel restriction-endonuclease site to allow a rapid identification of mutants [15]. The single-letter code for aa is used to name the human CD36 mutants. The number refers to the position in the CD36 primary sequence of the first residue mutated. The corresponding mutations are given after the solidus ('slash'). All mutations were confirmed by DNA sequencing.

COS-cell transfection

COS-cell monolayers at 50% confluence in six-well plates were transfected with 2 μg of CsCl-purified DNA and 9 μl of LipofectAmine reagent according to the manufacturer's instructions.

Abbreviations used: mAb, monoclonal antibody; aa, amino acid(s); f.a.c.s., fluorescence-activated cell sorting.

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mAbs

In all, seven murine mAbs specific for human CD36 were used in this study. OKM5 was obtained from Ortho Diagnostics (Raritan, NJ, U.S.A.). FA6-152 from Immunotech (Luminy, France). mAbs L103, 5F1 and SMΦ were gifts from the Fourth Leukocyte Workshop (Boston, MA, U.S.A.). mAbs 10/5 and 13/10 were produced and partially characterized in our laboratory (L. Daviet and J. L. McGregor, unpublished work).

Flow cytometry

Cells transfected with cloned DNA were detached with 5 mM EDTA 48 h post-transfection, washed in PBS and resuspended at a concentration of 1×10^6 cells/ml in PBS/1% BSA. Anti-CD36 mAbs were added at a concentration of 2.5 μg/ml for purified mAbs or at a dilution of 1:100 for ascites and then incubated for 1 h at 4 °C. After two washes in PBS, cells were incubated for 1 h at 4 °C with goat anti-(mouse IgG+IgM) antibodies fluorescein isothiocyanate conjugate. After a further series of washes, the level of CD36 expression was immediately assessed by fluorescence-activated cell sorting (f.a.c.s.) without cell fixation. Sample analysis was carried out on a Becton–Dickinson Facscan using the Lysis software. Propidium iodide (2 μg/ml) was added to each sample before flow-cytometric analysis to 'gate out' non-viable cells.

RESULTS AND DISCUSSION

The present study identifies an immunodominant structural/functional site on CD36 using human–mouse chimaeric CD36 constructs and homologue-replacement mutagenesis. Several lines of evidence support the above statement: (1) mAb OKM5 and other mAbs (FA6-152, L103, 5F1 and SMΦ) are known to bind to functional sites on CD36 (see below). (2) Analysis of a series of chimaeric exchanges between human and mouse CD36 localizes the epitopes of these functional mAbs to the CD36 domain that spans aa 155–183. (3) The key residues of the binding sites were further delineated to aa 152 (mAb L103), 156–157 (mAb OKM5) and 163 (mAbs FA6-152) using homologue-replacement mutagenesis.

mAb OKM5 binding to CD36 has been shown to inhibit thrombospondin binding [1], malarial cytoadhesion [3], CD36-mediated uptake of oxidized low-density lipoprotein [4] and macrophage recognition of apoptotic neutrophils [16]. Inhibition of these cell-adhesive functions by the same mAb suggests that the OKM5 epitope and the domains that mediate these events may be closely related. The functional properties of mAbs FA6-152, L103, 5F1 and SMΦ have also been well characterized. These mAbs totally (FA6-152, L103) or partially (5F1 and SMΦ) inhibit malarial cytoadhesion [17] and block CD36–oxidized low-density lipoprotein interaction (except SMΦ) [18]. The recognition and uptake of aged neutrophils by macrophages is inhibited by mAbs SMΦ and 5F1, but not by FA6-152 and L103 [16]. The common functional properties shared by these different mAbs suggest that they may recognize adjacent epitopes in a functional immunodominant region of the molecule.

The murine homologue of CD36 shows 85% aa identity with the human protein. The majority of the differences are conservative, and there are no deletions or insertions [4]. Despite the high degree of sequence similarity between the two proteins, OKM5 does not recognize mouse CD36 [4]. In order to determine if the other mAbs specific to human CD36 are able to cross-react with the murine protein, mouse platelet flow cytometry was performed [19] using saturating concentrations of the anti-human CD36 mAbs. The panel of mAbs specific for human CD36 used in this study failed to recognize mouse CD36 (results not shown). This property has been exploited to map regions of the protein important for mAb binding. A series of human–mouse CD36 chimaeras were constructed in which segments of human CD36 were systematically replaced with analogous sequences from murine CD36 (Figure 1). Such homologous domain substitutions should not disrupt the native conformation of the molecule, as genetically related proteins have similar three-dimensional structures [15,20,21].

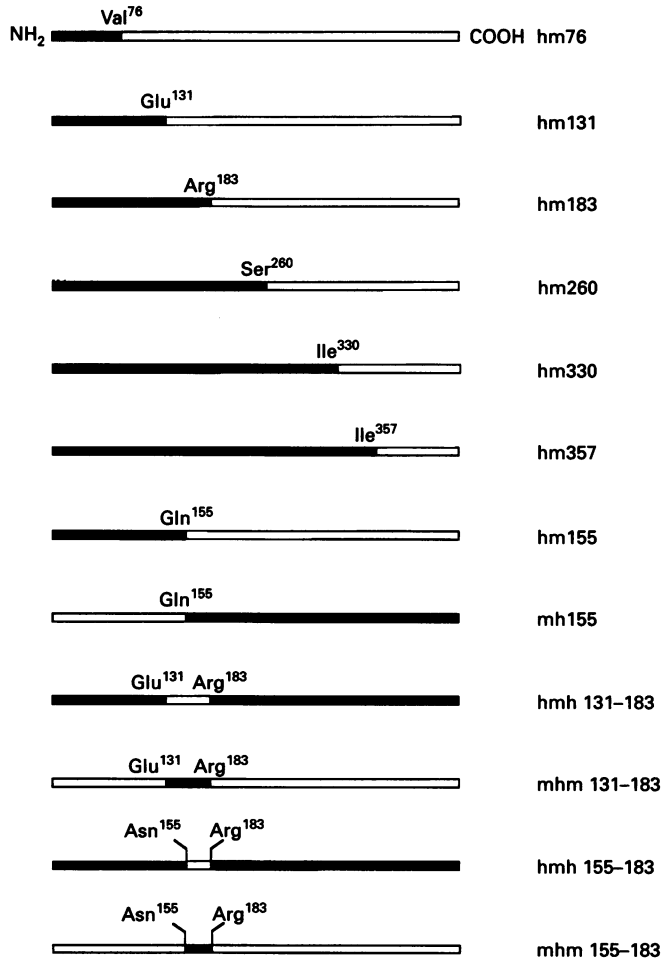

Figure 1 Schematic diagram showing the chimaeric constructs of CD36

The human–mouse chimaeras consist of human CD36 domains (closed bar) linked to the corresponding fragment of mouse CD36 (open bar). Some of the mutants were produced by cassette mutagenesis where human or murine segments were substituted with the homologous sequence from the other species. The positions of the junctions are indicated.

The chimaeric molecules were transiently expressed in COS cells and analysed by f.a.c.s.. Since none of the mAbs react with mouse CD36, the binding of mAbs OKM5, FA6-152, L103, 5F1, SMΦ and 10/5 to the hm183 chimaeric construct, but not to hm131, localizes their epitopes to the domain of aa 131–183 (Table 1). Specific recognition of the 131–183 domain by the members of the 'OKM5 cluster', mAbs OKM5, FA6-152, L103, 5F1, SMΦ and 10/5, was further confirmed by the specific binding of these mAbs to a CD36 chimaera in which the human 131–183 cassette (mhm131–183; Table 1) substitutes the homologous murine sequence. In contrast, binding of these mAbs to a human CD36 construct was abolished when the murine 131–183

Table 1 Binding of mAbs to CD36 chimaeras

COS cells were transfected with cDNA constructs and analysed by f.a.c.s. 48 h post-transfection. mAb binding is expressed as the percentage of cells staining above background, which shows some variation, since this value is affected by the transfection efficiency.

	mAb binding (%)						
CD36 construct	OKM5	FA6-152	L103	SMΦ	5F1	10/5	13/10
Human CD36	42	40	39	32	26	42	39
hm76	< 1	< 1	< 1	< 1	< 1	< 1	10
hm131	< 1	< 1	< 1	< 1	< 1	< 1	42
hm183	46	45	42	25	22	42	36
hm260, hm330, hm357	20	22	20	10	10	19	16
hm155	< 1	< 1	< 1	< 1	< 1	< 1	15
mh155	35	36	22	21	27	26	< 1
hmh131–183	< 1	< 1	< 1	< 1	< 1	< 1	17
mhm131–183	32	31	30	22	24	20	< 1
hmh155–183	< 1	< 1	< 1	< 1	< 1	< 1	12
mhm155–183	36	38	6	17	20	33	< 1

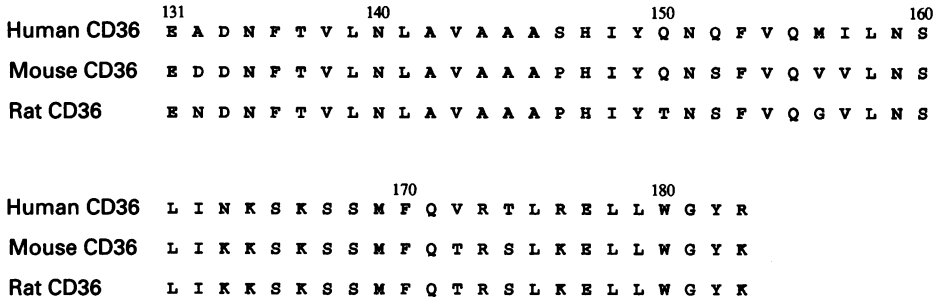

Figure 2 Alignment of human, mouse and rat CD36 sequences at the 131–183 region

The homologue-replacement mutations produced within the human 155–183 domain are Q152/S, M156I/VV, N163/K, V172/T, T174/S, R176/K and R183/K.

cassette replaced its human equivalent (hmh131–183; Table 1). To study this immunodominant region further, we constructed two chimaeras (hm155 and mh155; Figure 1). The binding of the OKM5 cluster to chimaera mh155, but not to hm155, localizes the epitopes of these mAbs to the 155–183 segment. Moreover, insertion of the human 155–183 domain into murine CD36 (mhm155–183) allows it to bind the OKM5 cluster. This construct may partially affect the epitope of mAb L103, however, since the percentage of L103-positive cells is inferior to that observed for the other mAbs (Table 1). The reverse construction, the insertion of a murine cassette into the human CD36 gene (hmh155–183), completely abolishes binding of the OKM5 cluster. A single mAb (13/10) binds specifically to sequence 1–76, which can be further narrowed to aa 30–76, since the uncleaved CD36 signal peptide is predicted to be embedded in the plasma membrane [22].

To map the mAb-binding determinants within the 155–183 domain, mutants were constructed using the principle of homologue-replacement mutagenesis, in which human residues were systematically substituted for the corresponding mouse residues throughout this region of the protein (Figure 2). An exception to this is the homologue-replacement mutation Q152/S, which was added because of its close proximity to the 155–183 segment. The mutated cDNAs were transfected into COS cells, and their expression analysed by f.a.c.s. using the panel of anti-CD36 mAbs. mAb 13/10 has been shown to recognize a site in the N-terminal part of the molecule. In Table 2, staining with mAbs OKM5, FA6-152, L103 and 10/5 is expressed relative to the percentage of 13/10-positive cells in the same transfection. All of the mutants were expressed on the cell surface, with 10–40% positive cells in each experiment. Since the percentage of positive cells obtained with the two mAbs of the IgM isotype (5F1 and SMΦ) was consistently lower, and therefore not comparable with that of the reporter mAb (13/10, IgG2b), the binding of these mAbs to the homologue-replacement mutants is not shown. The isotype difference may explain the lower affinity of these mAbs for CD36. The replacement of methionine-156 and isoleucine-157 by valine in both positions (M156I/VV) virtually eliminated OKM5 binding (Table 2). Since none of the other epitopes were disrupted by this mutation, it is likely that the effect observed on the binding of OKM5 is indicative of a specific disruptive effect by the aa substitutions rather than a conformational alteration of the domain. We can speculate that isoleucine-157 is a key residue of the OKM5 epitope, as this aa has often been shown to be found in the functional epitopes of murine mAbs [23]. According to predictions of CD36 secondary structure, these residues (M156I) are located in the central part of a β-strand comprising aa 152–161. Mutation N163/K completely inhibits the binding of two mAbs, FA6-152 and 10/5 (Table 2). This mutation replaces the glycosylated asparagine residue at position 163 with lysine. Of the ten N-glycosylation sites present on human CD36, this is the only one which is absent in the murine molecule. The effect on the binding of FA6-152 and 10/5

Table 2 mAb binding to CD36 mutants

The mutants were constructed using the principle of homologue-replacement mutagenesis. The single-letter code for amino acids is used to name the human CD36 mutants. The number refers to the position in the CD36 primary sequence of the first residue mutated. The corresponding mutations are given after the solidus ('slash'). Mutants were transfected into COS cells by the LipofectAmine technique and analysed by f.a.c.s. with the panel of anti-CD36 mAbs. The binding of the OKM5 cluster is expressed relative to control staining of the same population with 13/10. Values in bold show significant loss of mAb binding.

	mAb binding (% relative to 13/10)			
CD36 mutant	OKM5	FA6-152	L103	10/5
Q152/S	122	116	**26**	117
M156I/VV	**13**	109	108	111
N163/K	100	**0**	107	**0**
V172/T	125	132	124	120
T174/S	104	114	107	105
R176/K	114	120	108	110
R183/K	116	112	94	85

could be the consequence of a local conformational change, due to the absence of the carbohydrate structure, or of a direct involvement of the asparagine residue. The mutation Q152/S specifically affects L103 binding, which is consistent with the reduced binding observed for the mhm155–183 construct. Since none of the other mutations affect L103 binding, it is likely that additional conformational differences between human and mouse 155–183 domains confer specificity to human CD36. These results show that mAbs OKM5, FA6-152 and L103 possess specific contact determinants within the short immunodominant 155–183 domain. This could explain why these mAbs share specific functional properties.

A peptide derived from human CD36 aa 139–155 was previously reported to represent part of the OKM5 epitope [13]. Results reported in the present study, however, show that the 155–183 domain forms a key part of the OKM5 binding site: (1) substitution of human domain 155–183 with the murine homologous sequence (hmh155–183 construct) totally inhibits the binding of OKM5, even though the human 139–155 domain is present in the chimaeric molecule; (2) the presence of the human 155–183 segment together with the 139–155 domain derived from mouse sequence (mhm155–183 construct) supports the binding of OKM5; (3) the binding of OKM5 is only disrupted by the M156I/VV mutation, whereas the nearby aa substitutions Q152/S and N163/K have no effect. Our approach ensured a full examination of CD36 domains and largely eliminated effects due to conformational changes. Such a consideration is important, since the OKM5 epitope has been shown to be conformational [24]. The interface between an antibody and its antigen has been shown to be 2.5–3.0 nm (25–30 Å) in diameter [25]. The mutations generated in this study affect key residues of the OKM5 epitope, but not necessarily all the contact residues. We cannot exclude the possibility that other contact residues are present in the adjacent 139–155 sequence. The differences between the results reported in this study and those of Leung et al. [13] reflect the different strategies used and are not in contradiction. The OKM5 epitope is known to be discontinuous and may comprise residues present not only in 155–183 but also in 139–155. Leung et al. [13] do report in their study that 139–155 may represent part of the OKM5 binding site. It is of noteworthy that the 139–155 sequence is identical in both the human and mouse proteins, except for residues 146 and 152 (Figure 2). Taken together these results suggest that OKM5 binding is dependent on the 155–183 region, but residues in the adjacent 139–155 sequence may be important for such binding to occur.

In conclusion, we have localized a CD36 domain and identified determinants important for the binding of functional anti-CD36 mAbs. Our results suggest that the 155–183 domain represents an immunodominant region of CD36 in the mouse, since six out of seven anti-CD36 mAbs tested bind to this sequence. Moreover, antibody-blocking data suggest that this segment may be directly involved in CD36-mediated cell-adhesive functions. Further functional studies are in progress to verify this hypothesis.

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