The effects of cysteine to alanine mutations of CD18 on the expression and adhesion of the CD11/CD18 integrins

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Abstract Of the 56 cysteines in the extracellular domain of the CD18 antigen (β2 integrin subunit), corresponding ones are not found in 12 positions in the β4, β7, or β8 integrin subunits. These 12 cysteines were mutated to alanines, either singly or in pairs, in CD18. All these mutants can support the expression of all three CD11/CD18 integrins. Transfectants expressing these variant integrins are generally more adhesive than the wild-type, suggesting that the cysteine residues, perhaps by engaging in disulphide bonds, may contribute to the maintenance of the CD11/CD18 integrins in a resting state.

Key words: Cysteine to alanine mutation; CD11/CD18 antigen; Integrin; Disulphide bond

1. Introduction

The integrins are heterodimeric adhesion receptors formed by the non-covalent association of an α and a β subunit, both of which are type I membrane proteins [1,2]. Both the α and β subunits are involved in ligand binding [2]. Using the β2 integrins as an experimental system, we aim to determine the contribution of the β subunit in ligand binding and its regulation. The extracellular domains of the β subunits are organised linearly, from the N-terminal, a PSI (plexin–semaphorin–integrin) domain [3], a highly conserved region (HCR) with an I-domain like structure [4–6], a mid-region and the major cysteine-rich (CRR) region. Mutations in the HCR of the β2 [7–10] and β3 subunits [11] had led to the expression of integrins with no adhesion activities, suggesting that the HCR may directly participate in ligand binding. The CRR, on the other hand, may have a regulatory role. Deletion of the CRR in the β2 [12] and β3 [13] subunit does not affect the formation of the αLβ2 and the αIIbβ3 integrin, respectively. Furthermore, these integrins were shown to be constitutively active in ligand binding. It thus appears that the cysteine residues of the integrin β subunits, particularly those in the CRR, may have key roles in the regulation of integrin activation. We therefore mutated a set of cysteine residues in the β2 integrin subunit into alanines, and studied these mutants for their ability to form functional leukocyte function-associated antigen (LFA)-1 (αLβ2, CD11a/CD18), Mac-1 (αMβ2, CD11b/CD18), and p150,95 (αXβ2, CD11c/CD18) integrins.

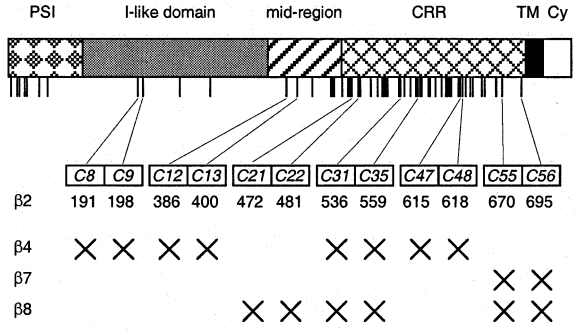

Fig. 1. The putative domain organisation of the integrin β subunits. The boundaries between different regions in the extracellular domain must remain approximate since, to date, they have not been fully characterised. The location of the 56 extracellular cysteines in the β1, β2, β3, β5 and β6 subunits is marked by vertical lines underneath the main diagram. For convenient and universal reference of the cysteine residues among the integrin β subunits, they are designated *C1* and *C56* from the N-terminal. The cysteine residues not found in β4, β7, and β8 are shown in boxes. The locations of these residues in the β2 subunit are shown underneath (the initiation methionine residue assigned as residue 1). Cysteine residues missing in β4, β7, and β8 are marked with crosses. TM, transmembrane segment; Cy, cytoplasmic segment.

Alignment of the extracellular sequences of the eight human β integrin subunits revealed 56 cysteine residues which are absolutely conserved in the β1, β2, β3, β5, and β6 subunits (Fig. 1). For convenient and universal reference, the cysteine residues will be referred to as *C1* to *C56* from the N-terminal end in this article. The other three β subunits lack the full complement of the 56 cysteines: β4, β7, and β8 have 48, 54, and 50 cysteines, respectively. The missing cysteines in these subunits do not appear to be random since the same pairs of cysteines are missing from two or more β subunits. *C55* and *C56* are missing from β7. In addition to *C55* and *C56*, β8 also lacks *C21*, *C22*, *C31*, and *C35*. *C31* and *C35* are also missing

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Abbreviations: mAb, monoclonal antibody; ICAM, intracellular adhesion molecule; LFA, leukocyte function-associated antigen; HCR, highly conserved region; CRR, cysteine-rich region

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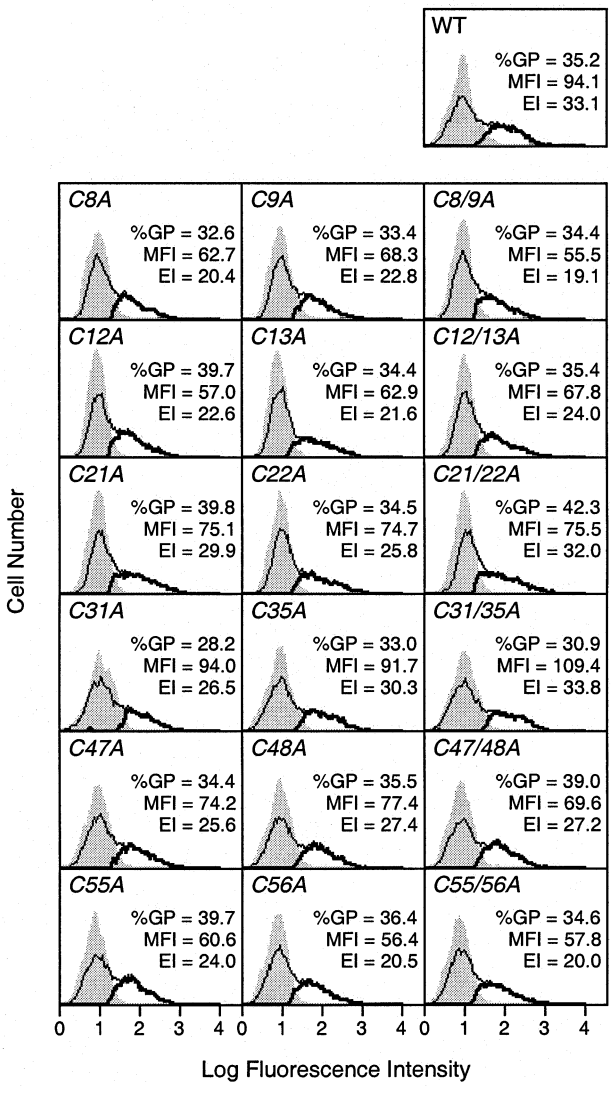

Fig. 2. Expression of LFA-1 (CD11a/CD18 antigen or αLβ2 integrin) on COS-7 transfectants. Wild-type and mutant CD18 cDNA were co-transfected into COS-7 cells with CD11a cDNA. Expression of the LFA-1 was monitored by flow cytometry using the mAb IB4 (solid lines). Background histograms (shaded) were obtained with irrelevant mAb OX34 of the same IgG2a isotype. Specific expression (shown in bold) is obtained by subtractive histogram analysis using the CellQuest software (Becton Dickinson) from which % gated positive cells (%GP) and mean fluorescent intensity (MFI) of the positive population were also obtained. The expression index (EI) is calculated as 100×(% positive cells)×MFI.

from β4, whose other six missing cysteines are *C8*, *C9*, *C12*, *C13*, *C47*, and *C48*. Since it is not possible to study the effect of all 56 cysteine residues at the same time, we decided to start with this set of 12 cysteine residues, with the rationale that at least some of them, because of their absence in other integrin subunits, may not be absolutely required for the expression of the β2 integrins.

2. Materials and methods

The monoclonal antibodies (mAbs) MHM24 (anti-αL), LPM19c (anti-αM) and KB43 (anti-αX) were obtained as previously described [10]. IB4 was purchased from ATCC. MEM48 was a gift from V. Horejsi (Institute of Molecular Genetics, Prague, Czech Republic). OX34 was a gift from M. Muklavec (Sir William Dunn School of Pathology, Oxford, UK).

The αL, αM and αX cDNA expression clones, in pcDNA3 (Invitrogen BV, Groningen, The Netherlands), were described previously [10,14]. The wild-type cDNA clone for the β2 integrin subunit was described in Douglass et al. [15]. All cysteine to alanine mutations were constructed using the strategy described by Horton et al. [16] with standard molecular biology techniques. All constructed cDNA clones were verified by sequencing.

COS-7 cells were transfected with the cDNA of various combinations of α and β subunits of the CD11/CD18 integrins and studied for their expression and adhesion to ligands with protocols described previously [10,12]. Expression of the CD11/CD18 integrins was determined by flow cytometry using the heterodimer specific mAb IB4 [17]. MEM48 [18], at 10 μg/ml, was added to promote adhesion.

3. Results

3.1. Expression of wild-type and variant LFA-1 on COS-7 transfectants

Wild-type β2 cDNA, or β2 cDNAs with single or double C→A substitution were co-transfected into COS-7 cells with the wild-type αL cDNA. Surface expression of LFA-1 was detected by flow cytometry using the heterodimer specific mAb IB4. The results are shown in Fig. 2. In all cases, LFA-1 expression was not significantly affected by the C→A mutations.

The transfectants were tested for their adhesion to immobilised intracellular adhesion molecule (ICAM)-1 (Fig. 3).

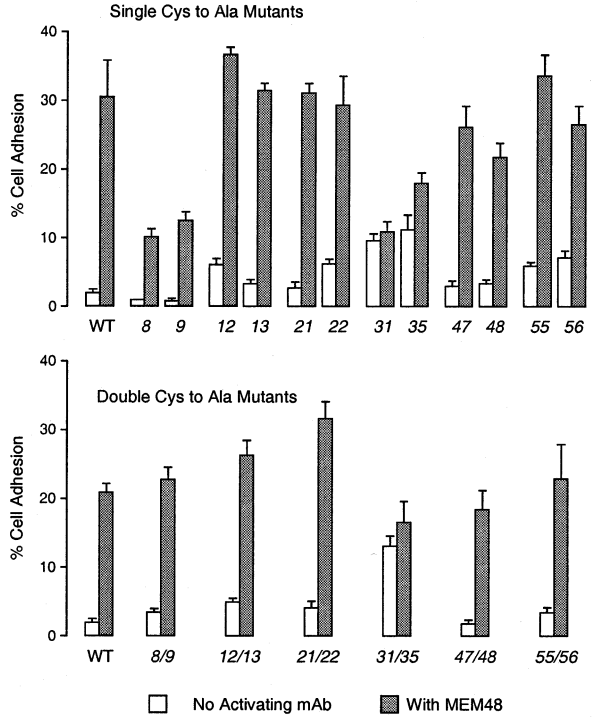

Fig. 3. Adhesion of COS-7 transfectants expressing wild-type LFA-1 and LFA-1 with single (upper panel) and double (lower panel) C→A mutations to ICAM-1. Mutated cysteines are indicated by the consensus cysteine numbering of integrin β subunits (see Fig. 1). Experiments were performed in RPMI media without activating reagents (open bars), and in the presence of the activating mAb MEM48 at 10 μg/ml (shaded bars). Data shown in the upper panel are obtained from a single representative experiment as are those shown in the lower panel. Adhesion can be blocked with the anti-αL mAb MHM24 (data not shown).

Transfectants expressing wild-type LFA-1 adhered poorly to ICAM-1 unless they were activated with the mAb MEM48. LFA-1 with the single C→A mutations also supported adhesion to ICAM-1 with a varying degree of efficiency: the least adhesive were the *C8A* and *C9A* variants, and the most adhesive were the *C31A* and *C35A* variants. The double C→A mutants showed, generally, a similar adhesion profile to their corresponding single C→A mutants. The adhesion of all LFA-1 variants was promoted to a higher level with the activating mAb MEM48. The most active group, namely, those with the single or double *C31A* and *C35A* mutations, were least responsive.

3.2. Expression of wild-type and variant Mac-1 and p150,95 on COS-7 transfectants

The six double C→A CD18 variants were also studied for their support of Mac-1 and p150,95 expression (Fig. 4), and adhesion to denatured BSA (Fig. 5). Similar results were obtained as for LFA-1.

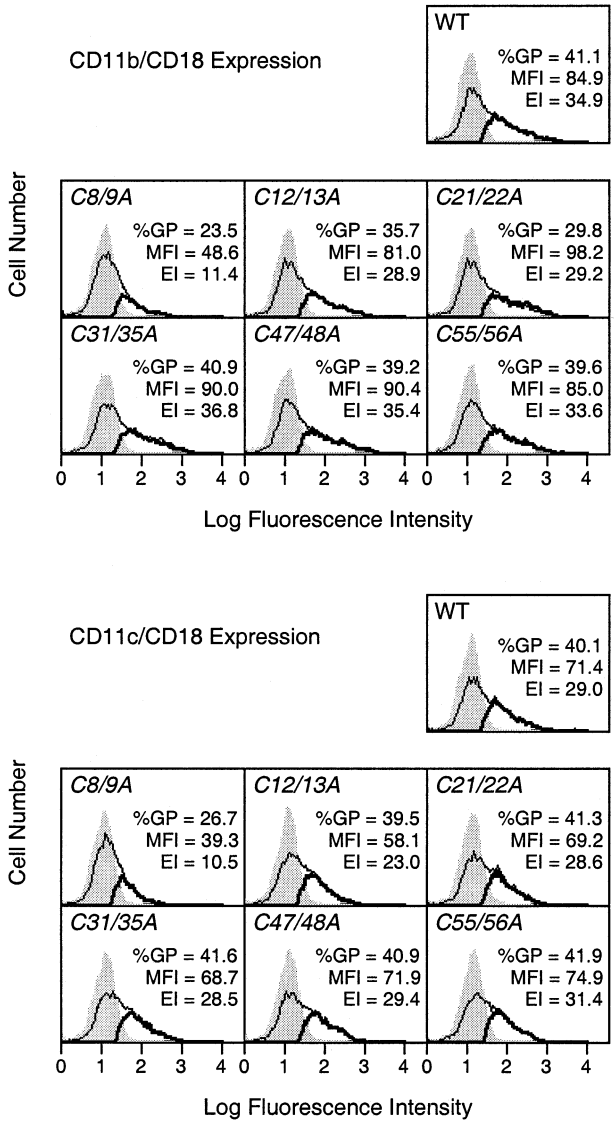

Fig. 4. Expression of Mac-1 (CD11b/CD18 antigen or αMβ2 integrin) and p150,95 (CD11c/CD18 antigen or αXβ2 integrin) on COS-7 transfectants. Wild-type and double C→A mutant CD18 cDNA were co-transfected into COS-7 cells with CD11b (upper panel) or CD11c (lower panel) cDNA. Expression of the Mac-1 and p150,95 antigens was monitored by flow cytometry using the mAb IB4 as described in Fig. 2.

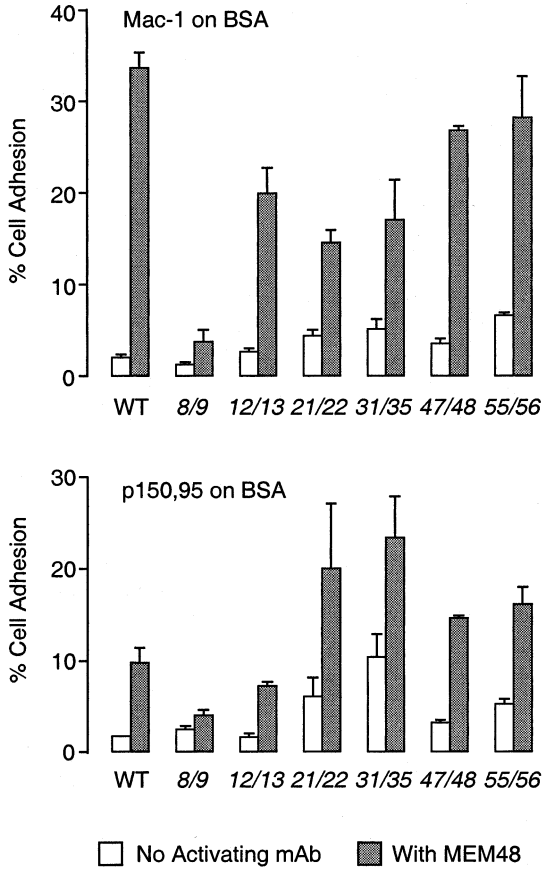

Fig. 5. Adhesion of COS-7 transfectants expressing wild-type Mac-1 and p150,95 and the integrins with double C→A mutant CD18 to denatured BSA. Details of the experiments are as in Fig. 3. Mac-1 and p150,95 adhesion can be blocked with the anti-αM mAb LPM19c and anti-αX mAb KB43, respectively (data not shown).

4. Discussion

The 56 cysteine residues, out of approximately 700 residues in the extracellular domains of the integrin β subunits, are arranged in a very characteristic pattern (see Fig. 1). Twelve of these cysteines, however, are not absolutely conserved, and can be grouped into six pairs. The importance of these cysteine residues in the β2 subunit was studied by mutation, either singly or in pairs, into alanine residues. All β2 variants with C→A mutations support the surface expression of LFA-1. Transfectants expressing LFA-1 also showed varying degrees of adhesion to ICAM-1, and adhesion can be promoted to a higher level with the activating mAb MEM48. The double mutants were also analysed for their capacity to combine with the αM and αX subunits. Transfectants were shown to express Mac-1 and p150,95 and to adhere to denatured BSA.

Taken together, these results showed that these cysteine residues are not required in the β2 integrin subunit for combining with the αL, αM and αX subunits, and for supporting the adhesion of the integrins to their respective ligands. On more detailed examination of the data from repeated experi-

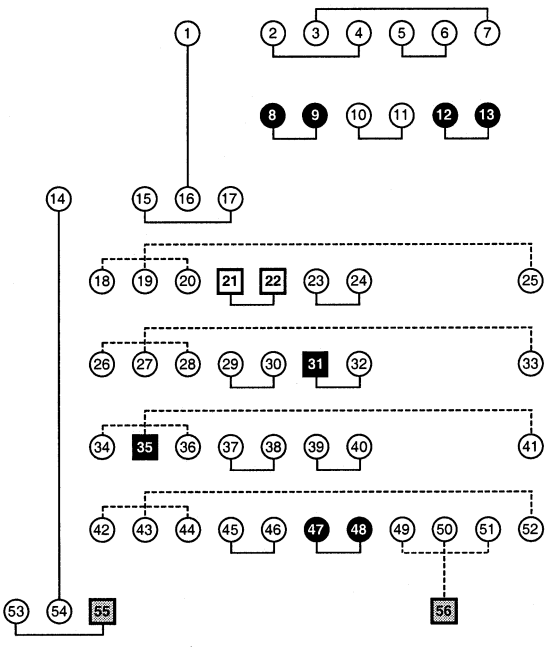

Fig. 6. The disulphide bonding model of the integrin β subunits. The model is based on the work of Calvette et al. [20]. The cysteine residues are labelled by consensus numbering from *C1* to *C56* from the N-terminal. Disulphide bonds are shown as solid lines between the residues; indeterminable and alternative assignments are shown as dashed lines. Cysteines missing in β4 are shown on black background, those missing in β7 on shaded background, and those missing in β8 are shown in square outlines.

ments, a general trend may be observed: the mutants within each pairing group behave similarly. Thus, the mutants in the *C8*/*C9* group are least effective in supporting integrin heterodimer expression, and expressed integrins are least adhesive to ligands. Those in the *C31*/*C35* group, on the other hand, are the most adhesive, and adhesion to ligand is easily detected in the absence of the activating mAb. It has been proposed that when a pair of cysteines are missing in homologous proteins, they may, when present, form a disulphide bond [19]. The present results are consistent with the proposed pairs of cysteines being engaged in disulphide bonds, since the main effect of the mutations of either or both may be regarded as the removal of the same disulphide bond. However, this cannot be regarded as definitive evidence.

A disulphide bonding model (Fig. 6) has been proposed for the β3 integrin subunit [20]. Because the CRR is resistant to proteolysis, disulphide bonds in the CRR were not determined biochemically, but were assigned based on the assumptions that there are four pseudo-repeating elements in the CRR and the disulphide bonds are self-contained within each repeating unit. Four of the six pairs of cysteines addressed in this work are also predicted to form disulphide bonds in this model. They are *C8–C9*, *C12–C13*, *C21–C22*, and *C47–C48*. The other two pairs, namely *C31* and *C35*, and *C55* and *C56*, were not predicted to engage in disulphide bonds with each other. If we apply this model to β4, β7, and β8, we are forced to conclude that either there are unpaired cysteines in these subunits, or their disulphide bonding patterns are different from that of β3. Alternatively, the disulphide bond assignments may have to be revised.

Previously, by replacing the CRR of the β2 subunit with that from β1, we showed that the resultant chimeric integrin β subunit combines with the αL subunit to form a LFA-1 that is constitutively active in ICAM-1 adhesion [15]. Together with the observations that many activating mAbs are mapped to the CRR, we postulated that the CRR is important in maintaining the integrin in its resting state. This is achieved possibly by the interaction between different regions in the CRR and/or their interaction with other regions of the integrin heterodimer, both in the β and the α subunit. By introducing an incompatible region from another integrin β subunit, or by the binding of an antibody, the normal interactions between the different regions are perturbed resulting in the release of the integrins into an active state. In the experiments presented here, the mutants involved *C12*/*C13*, *C21*/*C22*, *C31*/*C35* and *C55*/*C56* are more active than the wild-type β2. It is possible that these cysteine residues, perhaps by way of engaging in disulphide bonds, may contribute to the maintenance of the β2 integrins in their resting state.

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