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Molecules and Cells

Characterization of the Residues of αX I-Domain and ICAM-1 Mediating Their Interactions

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Integrin αXβ2 performs a significant role in leukocyte functions including phagocytosis and migration, and binds to a variety of ligands, including fibrinogen, iC3b, and ICAM-1. A particular domain of the α subunit of the integrin - the αX I-domain - is a ligand binding site, and the interaction of the αX I-domain and ICAM-1 on the endothelium is an important step in leukocyte extravasation. In order to elucidate the structural aspects of this interaction, we defined the moieties of the αX and ICAM-1 relevant to their interaction in this study. It was determined that the ICAM-1 binding sites of the αX I-domain were located in the α3α4, βDα5, and βFα7 loops at the top surface of the I-domain. The residues Q^{202}, K^{242}, K^{243}, E^{298} and D^{299} on these loops were crucial for the recognition of ICAM-1. Among these residues, K^{242} and K^{243} on the βDα5 loop were found to be the most salient, thereby suggesting an ionic interaction between these proteins. Domain 3 of ICAM-1 was identified as a primary binding site for the αX I-domain. Two regions of domain 3 (D^{229}QRLNPTV and E^{254}DEGTQRL) perform critical functions in the binding of the αX I-domain. Especially, the residue E^{254}DEG, is most important with regard to the αX I-domain.

INTRODUCTION

The β2 integrins are leukocyte-specific transmembrane proteins that integrate the extracellular matrix and the cytoskeleton. These molecules are heterodimeric proteins that perform critical roles in leukocyte functions such as adhesion, spreading, migration, and phagocytosis (Arnaout et al., 2005; Harris et al., 2000). The β2 integrins are composed of a common β subunit which associates with four other α subunits; αL, αM, αX, and αD (Arnaout, 2002; Plow et al., 2000). The majority of ligands for these integrins interact with a distinct motif of the α subunits, namely the I-domain, which consists of approximately 200 amino acids. A metal ion dependent adhesion site (MIDAS) is located on the top of the I-domain, which exhibits a unique three-dimensional structure with seven α-helices and six β-sheets. The loops interconnecting the helices and sheets near the MIDAS play a pivotal role in ligand recognition (Luo et al., 2007).

One of the β2 integrins, αXβ2 (CD11c/CD18, p150,95), is expressed abundantly on monocytes, macrophages, and dendritic cells (Myones et al., 1988: Shortman and Liu, 2002). Because of its abundant expression on most dendritic cells, αXβ2 serves as a dendritic cell marker (Metlay et al., 1990). Several ligands for αXβ2 have already been identified, including fibrinogen, plasminogen, Thy-1, iC3b, ICAM-1, ICAM-2, and ICAM-4 (Bilsland et al., 1994; Choi et al., 2005; Gang et al., 2007; Ihanus et al., 2007; Loike et al., 1991). αXβ2 may be involved in antigen presentation in dendritic cells and the adhesion of monocytes to the inflamed endothelium (Meunier et al., 1994; Sadhu et al., 2007).

Intercellular adhesion molecule-1 (ICAM-1, CD54) is an integral membrane protein with an extracellular region composed of five immunoglobulin-like domains, a transmembrane region, and a cytoplasmic tail (Lawson and Wolf, 2009). This protein is expressed on leukocytes, endothelial cells, and a variety of other cells, and plays an important role in immune-mediated cell-cell adhesion (van Buul et al., 2007). ICAM-1 binds to fibrinogen and most of the β2 integrins with the exception of αDβ2, and functions as a receptor for rhinovirus (Languino et al., 1993; Staunton et al., 1990).

The binding of the β2 integrin to ICAM-1 provides an important step for leukocytes to adhere to endothelium expressing ICAM-1, and subsequently to migrate out for the vascular system. Several studies have been conducted to demonstrate the nature of molecular interaction by defining the binding sites in both ICAM-1 and in the β2 integrin: the residues E^{34} and Q^{73} in domain 1 of ICAM-1 are essential for binding to αLβ2 (Stanley and Hogg, 1998) and the residues D^{229}QR and E^{254}DE in domain 3 of ICAM-1 are critical for αMβ2 recognition (Diamond et al., 1991).

Although the residues of ICAM-1 for αLβ2 and αMβ2 have already been studied in detail, the critical residues of ICAM-1 and αXβ2 mediating their interaction have yet to be clearly elucidated. Only one report thus far has implicated domain 4 of ICAM-1 in αXβ2 binding (Frick et al., 2005). In this study, we defined the critical residues of ICAM-1 and αXβ2 that mediate their interaction. The critical residues of the αX I-domain, which functions as a ligand-binding site of αXβ2, were determined and the regions of ICAM-1 responsible for αX I-domain binding were defined in detail.

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MATERIALS AND METHODS

Peptides and recombinant proteins

Several peptides were purchased from Peptron Co. (Korea). A set of peptides was derived from the amino acid sequences of the loops near the MIDAS of the αX I-domain: βAα1 (GSGSIS), α3α4 (HQLQGFTY), βDα5 (GKKEGDSLDYKD), βEα6 (GLAF-QNR) and βFα7 (EDFDALKDA). As a negative control, a peptide (GPRVVERHQSAC) was synthesized on the basis of a sequence derived from the human fibrinogen α-subunit of the central domain, which does not bind to ICAM-1. The other set of peptides was derived from the sequence of ICAM-1 domain 3: D3a (DQRLNPTV), D3b (TYGNDSF), D3c (EDEGTQRL), D3d (NQSQETLQ), D3a1 (DQRL), D3a2 (NPTV), D3c1 (EDEG), D3c2 (TQRL). Scrambled peptides were synthesized on the basis of mixed-up sequences of D3a, D3b, and D3c: D3as (from D3a: NRLPQTDV), D3bs (from D3b: SYDGTFNS) and D3cs (from D3c: LREQEGDT). Recombinant ICAM-1 fused with the human IgG Fc region was purchased from R & D Systems (USA).

Purification of I-domain and mutant proteins produced by *E. coli*

All of the αX I-domain mutant proteins were purified from bacterial clones harboring mutated αX I-domain cDNAs generated previously via site-directed mutagenesis as described previously (Lee et al., 2007). *E. coli* cells [Bl-21(DE3)] transformed with expression plasmids were incubated and induced with 0.5 mM Isopropyl-thio-β-D-galactoside (IPTG). The αX I-domain and the mutant proteins, all of which were glutathione S-transferase (GST) fusion proteins, were purified via affinity chromatography using a glutathione-Sepharose 4B column (GE Bioscience, USA).

Characterization of binding by surface plasmon resonance (SPR) analysis

SPR experiments were conducted on a Biacore X (Biacore, Sweden). All experiments were conducted at 25°C, and the analytes were diluted with HBS (150 mM NaCl, 100 mM HEPES, pH 7.4). ICAM-1 or αX I-domain was immobilized on a carboxyl-methyl dextran chip (CM5) at approximately 900 RU or 2000 RU, respectively, in accordance with the manufacturer's instructions. HBS with 1 mM $MgCl_2$ was utilized as a running buffer and 20 mM Tris/HCl (pH 8.0), 0.3 M NaCl, 20 mM diamine tetraacetic acid disodium (EDTA) were used for the removal of bound proteins. Analytes such as the αX I-domain or the synthetic peptides were injected for 90 s for association and for 100 s for dissociation at a speed of 20 μl/min. Binding kinetics were analyzed using BIAevaluation 3.0 software using a 1:1 Langmuir binding model. For peptide binding analyses, the binding levels of the injected peptides were calculated from the plateau regions of the association phase of each sensorgram.

Cell adhesion assay

ICAM-1-expressing HEK293 cells were generated by the transfection of a full-length ICAM-1 cDNA mixed with Lipofectamine 2000 in accordance with the manufacturer's instructions (Invitrogen, USA). ICAM-1-expressing cells were selected by G418 (1 mg/ml) and ICAM-1 expression levels were evaluated via flow cytometry analysis.

Purified αX I-domain (1 μM) was loaded on flat-bottom 96-well plates for immobilization. After incubation overnight at 4°C, the plates were washed with phosphate buffered saline (PBS) and blocked with 5% bovine serum albumin (BSA). ICAM-1-expressing cells previously labeled with 2 mg/ml of Calcein AM (BD Bioscience, USA) by 1 h of incubation in HBS with 1 mM $MgCl_2$ and 1 mM $CaCl_2$ at 37°C were added to each well at a concentration of 1×10^6 cell/ml (100 μl per well). Unbound cells were removed after 30 min of incubation at 37°C with or without peptides derived from the αX I-domain. The fluorescent levels of the bound cells on each plate were measured by spectrofluorometer (Spectramax, USA) with an emission wavelength of 530 nm.

Purification of soluble ICAM-1 domains produced by mammalian cells

cDNAs encoding the ICAM-1 leader peptide, domain 1 and 2, domain 3, domain 4, domain 4 and 5 of ICAM-1 were amplified by polymerase chain reaction. The leader sequence of ICAM-1 was inserted into pS521, which contains human immunoglobulin CH_2 and CH_3 gene (Schneider, 2000). The resulting plasmid was ligated with cDNA fragments encoding various ICAM-1 domains (domain 1 + 2, domain 3, domain 4, domain 4 + 5) to form several expression plasmids (pShID12, pShID3, pShID4, pShID45). These expression plasmids were then introduced into Chinease Hamster Ovary (CHO) cells by electroporation according to manufacturer's manual (Biorad, USA). Transfected cells were selected by Dulbeco's Modified Eagle's Medium (DMEM) with G418 (1 mg/ml) and further incubated in serum-free medium (HyQSFM4CHO, Hyclone, USA) to produce soluble ICAM-1-Fc recombinant proteins. Cell supernatant was collected and ICAM-1-Fc recombinant proteins were purified by an affinity chromatography using a Protein-G Sepharose column. Eluted recombinant proteins were dialysed against PBS and concentrated by Centricon (Amicon, USA).

Purification of ICAM-1 domain 3 produced by *Pichia*

cDNA encoding for the ICAM-1 domain 3 from pShID3 was introduced into pPICZαC, which is a *Pichia* expression vector that contains six histidine residues as a metal binding moiety (Invitrogen, USA). The resultant expression plasmid was introduced into *Pichia* cells via electroporation at 1.5 kV, 25 μF and 200 ohms. The treated cells were then plated on YPD plates with Zeocin (200 ng/ml) and incubated for 2 days at 30°C until colonies formed. Selected positive colonies were then incubated in yeast extract peptone dextrose medium (YPD) with Zeocin for 3 days and then further incubated in the presence of 0.5% methanol for induction. The cell supernatants were collected and subjected to affinity chromatography using a Ni-nitrilotriacetic acid (NTA) column in accordance with the manufacturer's instructions (Invitrogen, USA). The homogeneity of the isolated ICAM-1 domain 3 with His tag was evaluated via SDS-polyacrylamide gel electrophoresis (PAGE).

Binding analysis of ICAM-1 domains to αX I-domain

Microtiter plates were coated with 0.1 ml of the αX I-domain (1 μM) overnight at 4°C, then blocked with 5% BSA. The recombinant ICAM-1 domains with Fc (domain 1 and 2; D12-Fc; domain 3, D3-Fc; domain 4 and 5, D45-Fc) were added to each well to a final concentration of 1 μg/ml, then incubated for 1 h at 37°C. The plates were incubated for 1 h with anti-human IgG conjugated with alkaline phosphatase. The amount of ICAM-1 domains bound to the αX I-domain in each well was determined via absorbance measurements at 550 nm (Bio-Rad microplate reader 550, Bio-Rad, USA) after color reaction with the alkaline phosphatase substrate, Bluephos™ (KPL, USA).

Loops	Sequences
	βA α1
βA α1	**FLID** SGSISS **SRNF**
	α3 α4
α3α4	**LSLL** ASVHQLQGFTY **TATA**
	βD α5
βDα5	**IVIT** DGKKEGDSLD **YKDVIP**
	βE α6
βEα6	**AIGV** GLAFQ **NRNS**
	βF α7
βFα7	**IFKV** ED **FDALKDIQNQ**

Fig. 1. The amino acid sequences of five loops and loop-specific peptides of the αX I-domain. Peptide sequences marked by underbars were based on the loop sequences of the αX I-domain (Vorup-Jensen et al., 2003). Bold letters show the sequences of secondary structures linked to the loops.

Inhibition of GST-αX I-domain binding to ICAM-1 by domain 3 specific peptides

Microtiter plates were coated with the purified D3-Fc protein (1 μM), and were then blocked with 5% BSA. The αX I-domain (1 μM) was pre-incubated for 1 h with 1 mM of domain 3 specific peptides in a binding buffer (150 mM NaCl, 20 mM HEPES, 1 mM $MgCl_2$, pH 7.4), then added to each well (100 μl per well) for an additional 1 h of incubation. The amount of the αX I-domain bound to D3-Fc in each well was assessed by color reaction with alkaline phosphatase substrate after incubation with anti-GST antibody as well as anti-mouse IgG conjugated with alkaline phosphatase.

RESULTS

As a first step in defining the critical residues of the αX I-domain for ICAM-1, we attempted to determine the important loops of the αX I-domain responsible for ICAM-1 binding. Previous reports demonstrated that the loops at MIDAS were crucial for the binding of αXβ2 to its ligands such as fibrinogen and Thy-1 (Choi et al., 2005; Lee et al., 2007). Five peptides (βAα1, α3α4, βDα5, βEα6, and βFα7) were synthesized according to the sequences of the loops, which locate near MIDAS surface of the αX I-domain (Fig. 1). The binding activities of the peptides to ICAM-1 were evaluated via SPR analysis. As shown in Fig. 2, the βDα5, α3α4 and βFα7 peptides bind to ICAM-1 at a high level, whereas βEα6 and βFα7 peptide do not. This result suggests that the loops of the αX I-domain (βDα5, α3α4, and βFα7) are important for ICAM-1 recognition.

To confirm this result, a competitive inhibition experiment was conducted with loop-specific peptides and ICAM-1-expressing cells. A result of flow cytometry analysis (Fig. 3A) shows a high level of ICAM-1 expression on transfected HEK293 cells. And αX I-domain binding experiments with the loop specific peptides reveal that α3α4, βDα5, and βFα7 can inhibit the binding of the cells to the αX I-domain, whereas βAα1 and βEα6 cannot (Fig. 3B). The inhibitory activity of the βDα5 peptide appears to be higher than those has been detected in any other peptides. A combination of α3α4, βDα5, and βFα7 peptides completely blocks the binding of ICAM-1-expressing cells to the plates. These results indicate that the βDα5, α3α4, and βFα7 loops of the αX I-domain are the critical binding sites for ICAM-1.

To further delineate the important residues on the critical loops, we attempted to determine whether point mutations on the loops affected the binding of the αX I-domain to ICAM-1.

Table 1. Binding kinetics of αX I-domain mutants to ICAM-1

Mutant[1]	Loop	K_D (10^{-6} M)[2]
Wild type αX I-domain		0.73 ± 0.20
G141M	βAα1	1.42 ± 0.23
SISS142/5RIQP	βAα1	0.90 ± 0.29
S199A	α3α4	0.78 ± 0.15
Q202A	α3α4	3.46 ± 0.52
Q203/4GG	βDα5	0.37 ± 0.13
KK242/3AA	βDα5	4.17 ± 0.53
K243A	βDα5	2.50 ± 0.57
D249M	βDα5	0.42 ± 0.16
Y250A	βDα5	1.52 ± 0.36
K251A	βDα5	1.51 ± 0.35
D252A	βDα5	1.48 ± 0.21
Q274A	βEα6	0.35 ± 0.04
ED298/9ST	βFα7	2.92 ± 0.55

[1]The first letter indicates the amino acid residue at the position of the αX I-domain amino acid sequence. The second letter at the right hand side shows the changed amino acid after mutagenesis. All amino acids are shown as single letter codes.

[2]All data are shown as means ± S. E. of three experiments.

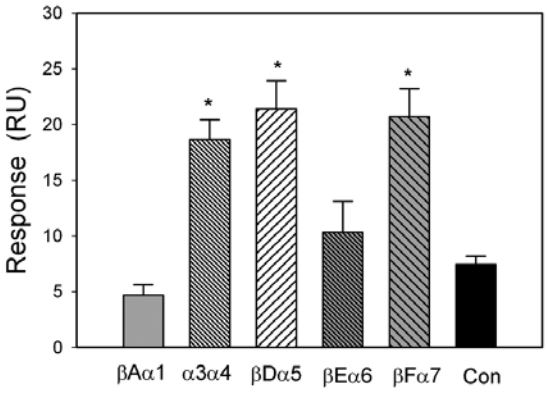

Fig. 2. Binding of loop-specific peptides to ICAM-1. ICAM-1-Fc (900 RU) was immobilized on a CM5 chip and the loop-specific peptides derived from the αX I-domain were injected to flow over the immobilized ICAM-1-Fc. Binding levels of the peptides are expressed as response unit (RU) values at the steady phase of the association curve of each peptide sensorgram. Results are expressed as means ± S. E. (n = 3). *, $p < 0.01$, significantly different from the control (Student's t test).

SPR analyses were conducted to determine the dissociation constants (K_D) of the αX I-domain mutants. We noted no significant changes in the binding affinity to ICAM-1 in many mutants (Table 1). However, mutant Q202A (in α3α4 loop), KK242/3AA and K243A (in βDα5 loop), ED298/9ST (in βFα7 loop) evidenced significant alterations in their K_D values: the affinities of these mutants were reduced to 1/3 to 1/6 of normal values. This result shows that the Q^{202}, K^{242}, K^{243}, E^{298}, and D^{299} residues are responsible for the binding of ICAM-1, and the K^{242} and K^{243} residues are most critical. These critical residues are either charged or polar amino acids, thus suggesting that the binding of the αX I-domain and ICAM-1 is mediated by an ionic interaction.

In the next stage, we attempted to define the ICAM-1 moieties responsible for the αX I-domain. Previously, Flick et al. (2005) reported that domain 4 of ICAM-1 was essential for binding to the αXβ2 integrin. Thus, we attempted to confirm the

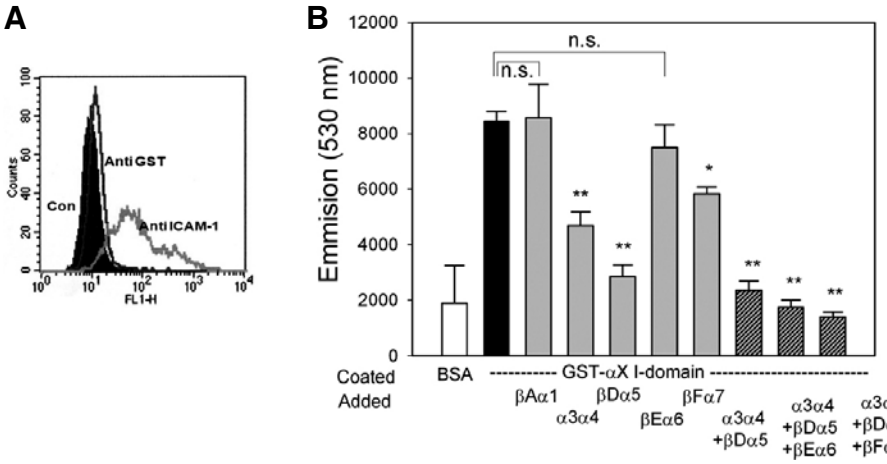

Fig. 3. Binding of ICAM-1 expressing HEK293 cells to the αX I-domain. (A) Expression of ICAM-1 on transfected HEK293 cells. A result of flow cytometry is depicted as a histogram with the log of the fluorescence intensity on the abscissa and the cell number on the ordinate. Stably transfected HEK293 cells with ICAM-1 expressing plasmid were incubated in the presence of anti ICAM-1 antibody or anti GST antibody as a control for 30 min at 4°C. The cells were washed, stained with fluorescein-conjugated goat anti-mouse Fc for 30 min, and analyzed on a FACScan. (B) The cells labeled with Calcein AM were preincubated with 1 mM of the peptides, then added to the αX I-domain coated plates. After 30 min of incubation, the numbers of adherent cells were assessed via emission fluorescence (530 nm). The data are expressed as means ± S. E (n = 3). *: $P < 0.05$ and **: $P < 0.01$ (Student's t test), n.s.: not significant ($P > 0.05$). with excess amounts of loop-specific peptides.

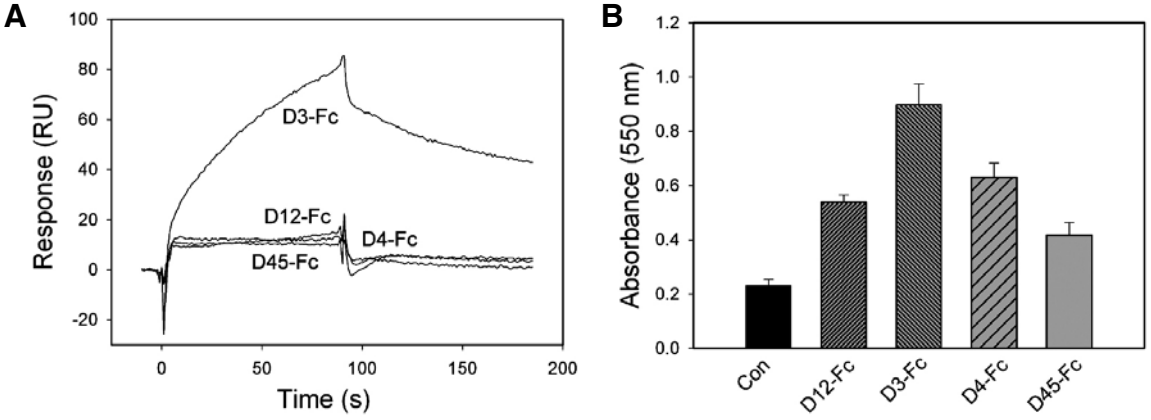

Fig. 4. Binding of purified recombinant ICAM-1 domain to the αX I-domain. (A) SPR sensorgram to show binding of ICAM-1 domains to the immobilized αX I-domain. Recombinant ICAM-1 domains fused with Fc (1 μM) were injected over a CM-5 chip with immobilized αX I-domain (2000 RU): domain 1 and 2, D12-Fc;domain 3, D3-Fc; domain 4, D4-Fc; domain 4 and 5, D45-Fc. (B) Binding of recombinant ICAM-1 domains to immobilized αX I-domain on microtiter plates (1 μg/ml). Recombinant ICAM-1 domains were added to each well (1 μM) and the binding levels were assessed by anti human Fc antibody. Nonspecific antibody was used as a control. Data are expressed as means ± S. E (n = 3).

binding activity of domain 4 of ICAM-1 to the αX I-domain and then to define the moieties of ICAM-1 responsible for the αX I-domain. Recombinant proteins (D12-Fc, D3-Fc, and D45-Fc) were purified for SPR and solid phase analyses. Counter to our initial expectations, D4-Fc did not bind to the αX I-domain, whereas D3-Fc did (Fig. 4A). The results of solid phase analysis also confirm that the binding level of D3-Fc to the αX I-domain is higher than the levels measured for other domains (Fig. 4B). These results led us to conclude that domain 3 of ICAM-1, not domain 4, is essential for the αX I-domain.

In order to define the interacting sites of domain 3, four sequence-specific peptides derived from domain 3 of ICAM-1 (D3a, D3b, D3c, and D3d) were tested for their direct binding activities for the αX I-domain (Fig. 5). The regions corresponding to these peptides were selected from the data regarding the three-dimensional structure of ICAM-1 domains 3 to 5 (Yang et al., 2004) and from a previous report showing αMβ2-binding regions on domain 3 of ICAM-1 (Diamond et al., 1991). The sequence-specific peptides, D3a and D3c, bind to the αX I-domain, whereas D3b, D3d, and D3cs do not (Fig. 6A). Because D3cs is a scrambled peptide of D3c, D3c binds to the αX I-domain in a sequence-specific manner. D3as, a scrambled peptide of D3a, binds slightly to the αX I-domain; however, the binding level of D3a is much higher than that of D3as, thereby indicating that the binding of D3a to the αX I-domain is also sequence-specific. To further define the binding sites of the D3a and D3c regions, shorter peptides with 4 amino acids (D3a1, D3a2, D3c1, and D3c2) were tested for the binding activity to the αX I-domain. In Fig. 6B, D3c1 peptide (sequence EDEG) derived from D3c appears to bind more than any other peptides. Taken together, these data illustrate that two regions of domain 3 (D^{229}QRLNPTV and E^{254}DEGTQRL) can interact with the αX I-domain and E^{254}DEG region is most important for the αX I-domain binding.

To confirm these results, we attempted to determine whether excess quantities of D3a and D3c peptides would inhibit the binding of D3-Fc to the αX I-domain. The binding level of D3-Fc is reduced to 70% and 50% of normal levels in the presence of D3a and D3c peptides, respectively (Fig. 7). The scrambled peptides (D3as, D3cs) evidenced no inhibitory effects. This

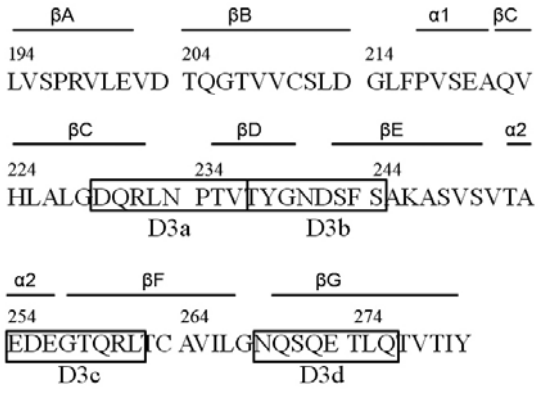

Fig. 5. Amino acid sequence of ICAM-1 domain 3. The domain 3-specific peptides are marked by boxes and the secondary structure of the domain 3 is marked above the sequence (Yang et al., 2004).

result shows that the D3c and D3a regions are critical for αX I-domain recognition.

To confirm that the D3c region of domain3 is involved in the recognition of the αX I-domain, a binding kinetic analysis was carried out with the αX I-domain and mutant domain 3 of ICAM-1 in which three amino acids $E^{254}DE$ in the D3 region were substituted to $K^{254}EK$. In previous report, this mutation was shown to inhibit purified αMβ2 binding to cell surface ICAM-1 (Diamond et al., 1991). This mutant protein was expressed and purified from *Pichia* as a His tagged protein and tested for its binding activity to the αX I-domain. As shown in Table 2, the dissociation constant of the mutant protein [D3-EDE254/6KEK-$(His)_6$] is about two to three times higher than that of wild type domain 3 [D3-$(His)_6$]. This low binding affinity of the mutant protein is caused by the K_{on} which is nearly one third of that for the wild type protein. This result suggests that the mutation ($E^{254}DE$ to $K^{254}EK$) inhibits the binding of the domain 3 to the αX I-domain. Since the other region of domain 3, such as D3a region, is also involved in the recognition for the αX I-domain, it is likely that the inhibitory effect of the mutation is not high. However, this result reinforces the notion that D3a1 region ($E^{254}DE$) is critical for αX I-domain.

In addition, the K_D value of wild type domain 3 produced by *Pichia* (1.35 μM) is two times higher than that of ICAM-1-Fc secreted by mammalian cells (0.73 μM, Table 1). The differences in K_D values of ICAM-1-Fc and D3-$(His)_6$ can be explained by dimerization of ICAM-1-Fc: this moelcule can form a dimer with its Fc fusion partner. It was reported that ICAM-1 normally existed as a dimer on cell surface and the dimerization of ICAM-1 caused about 1.5-3-fold increases of its affinity to ligands (Yang et al., 2004).

In conclusion, αX and ICAM-1 and the moieties relevant to their interaction were characterized in this study. ICAM-1 binding sites of the αX I-domain were located in the α3α4, βDα5, and βFα7 loops of the αX I-domain. The Q^{202}, K^{242}, K^{243}, E^{298}, and D^{299} residues on these loops perform an important role in the recognition of ICAM-1. Residues K^{242} and K^{243} on the βDα5 loop are the most critical of these residues. Domain 3 of ICAM-1 was identified as a primary binding site for the αX I-domain. Two regions of domain 3 (D^{229}QRLNPTV and E^{254}DEGTQRL), especially the residue E^{254}DEG, were found to be important with regard to the αX I-domain.

DISCUSSION

In this study we identified Q^{202}, K^{242}, K^{243}, E^{298}, and D^{299} as critical residues of the αX I-domain for ICAM-1 binding. These charged (K^{242}, K^{243}, E^{298}, and D^{299}) and polar (Q^{202}) amino acids have side chains that perform a crucial function in ligand recognition. The side chains of K^{242}, E^{298}, and D^{299} protrude from the MIDAS surface of the I-domain and do not interact with other residues of the I-domain (Fig. 8A). On the other hand, the side chains of Q^{202} and K^{243} are tilted toward adjacent α helices, and are involved in the formation of hydrogen bonds (Q^{202} and I^{143}, K^{243} and L^{248}). However, the portions of the loops in which these residues are located (Q^{202}, the α3 side of α3α4 loop; K^{243}, the α5 side of βDα5 loop) are quite flexible and movable, according to the crystal structure data of the αX I-domain (1N3Y). Therefore, it appears quite likely that side chains of Q^{202} and K^{243} can be released from intra-interaction and participate in ligand recognition, or vice versa.

There is a possibility that mutations may not be critically important for interaction between the αX I-domain and ICAM-1. Rather, the instability introduced by the mutations affected the

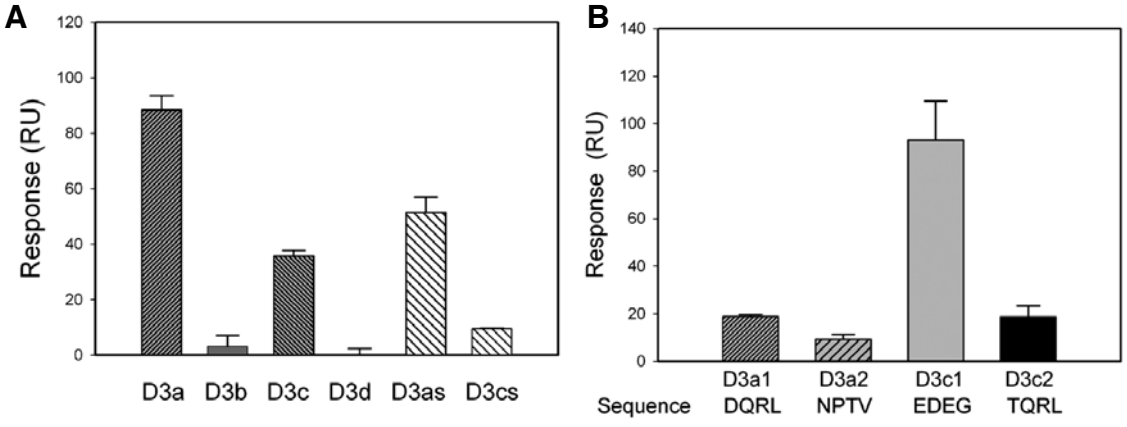

Fig. 6. Binding of ICAM-1 domain 3-specific peptides to the αX I-domain. αX I-domain was immobilized on a CM5 chip (2000 RU) and peptides derived from the domain 3 of ICAM-1 were injected to flow over the αX I-domain. Binding levels of the peptides are expressed as response unit (RU) values at the steady phase of the association curve of each peptide sensorgram. Results are expressed as means ± S. E. (n = 3). (A) Binding of domain 3-specific peptides with 8 amino acids (400 μM). (B) Binding of 4 amino acid peptides derived from D3a and D3c regions of the ICAM-1 domain 3 (1 mM).

Table 2. Binding kinetics of the αX I-domain to the domain 3 and the domain 3 mutant of ICAM-1

Analyte[1]	Ligand[2]	K_D (10^{-6} M)	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})
D3-$(His)_6$	GST-αX- I	1.35 ± 0.08	15,100 ± 2,710	0.020 ± 0.003
D3-EDE254/6KEK-$(His)_6$	GST-αX- I	3.54 ± 0.16	5,220 ± 750	0.019 ± 0.003

[1]Injected proteins for SRP analysis
[2]Proteins immobilized on CM5 chips
All data are expressed as means ± S. E. (n = 3)

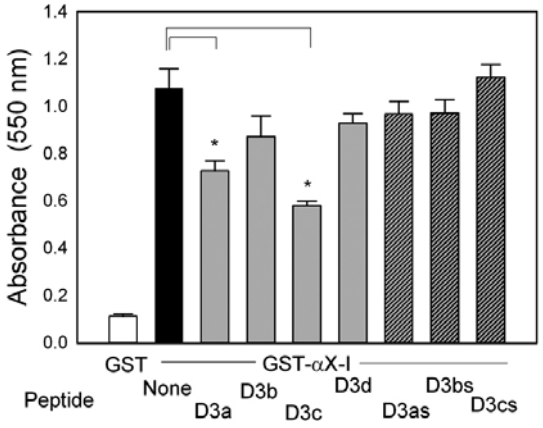

Fig. 7. Competitive inhibition of the domain 3-specific peptides for the binding of D3-Fc to the αX I-domain. The αX I-domain was immobilized on microtiter plates (1 μg/ml) and D3-Fc (1 μM) was incubated in the plates with the domain 3-specific peptides (1 mM). Data are expressed as means ± S. E. (n = 3). *, $P < 0.05$.

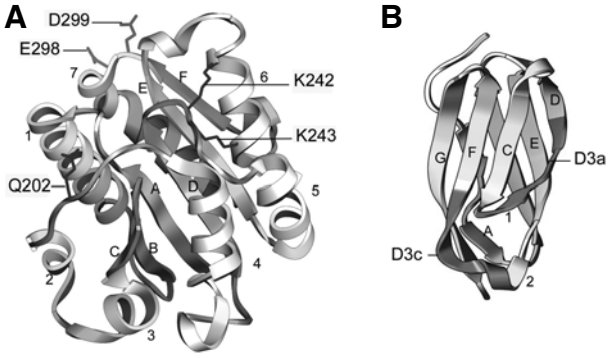

Fig. 8. Crystal structures of the αX I-domain (A) and domain 3 of ICAM-1 (B). These two structures were drawn by the UCSF Chimera computer program (Pettersen et al., 2004). A ribbon model of the αX I-domain is drawn on the basis of its crystal structure, PDB # 1N3Y (Vorup-Jensen et al., 2003). Five critical residues for ICAM-1 binding are shown (Q^{202}, K^{242}, K^{243}, E^{298}, D^{299}). A three-dimensional model of ICAM-1 domain 3 is from the crystal structure of ICAM-1 domains 3 to 5, PDB # 1P53 (Yang et al., 2004). Two binding sites for the αX I-domain are shown D3a (D^{229}-V^{236}) and D3c (E^{254}-R^{261}). In these models, α helices are denoted as Arabic numbers and β sheets are indicated as alphabet letters.

binding of the αX I-domain and ICAM-1. This possibility can be ruled out by two lines of evidence. First, we conducted a molecular dynamics simulation of the mutant proteins after energy minimization using a steepest descent method to estimate the structural perturbation induced by the mutations. The results demonstrated no significant alterations of the I-domain structure induced by the mutations, thereby suggesting that the mutant I-domains are stable in their wild-type form (data not shown). Because the critical residues for ligand recognition are either on flexible loops or are not involved in intra-interactions like hydrogen bond formation, it appears plausible that the mutations on these residues may not induce any structural perturbations or instability.

The other line of evidence for structural stability is derived from the binding experiments of these mutants to other ligands. The mutant Q202A binds to Thy-1 equally as well as the wild type αX I-domain: the K_D of wild type and Q202A is 1.16 ± 0.14 and 0.93 ± 0.26, respectively (Choi et al., 2005). The mutant KK242/3AA and the αX I-domain have the same level of binding affinity to RAGE (receptor for advanced glycation end products): the K_D values of the wild-type and KK242/3AA were 0.49 ± 0.03 and 0.62 ± 0.01, respectively. The mutant ED298/ 9ST and the αX I-domain bind JAM-3 (junctional adhesion molecule-3) were at the same levels: the K_D values of the wild-type and ED298/9ST were 0.29 ± 0.06 and 0.38 ± 0.04, respec tively (our unpublished observations). These same dissociation constant values strongly indicate that the mutant I-domains are as stable and functional as the wild type I-domain. Taken together, these two lines of evidence demonstrate that mutations introduced in the αX I-domain do not induce structural instability, but rather affect only the association of αX and ICAM-1.

In this study the most critical residues were K^{242} and K^{243}, which are located in loop βDα5 at the top of the αX I-domain. These basic residues may perform a critical role in an ionic interaction with the acidic amino acids of ICAM-1. This finding is consistent with data showing that the critical regions of domain 3 of ICAM-1 for the αX I-domain harbor acidic residues, most notably E^{254}DE. These basic residues, K^{242} and K^{243}, are also involved in the recognition of other ligands, such as fibrinogen and Thy-1 (Choi et al., 2005; Lee et al., 2007). Vorup-Jensen et al. demonstrated that the exposure of acidic residues on decayed proteins by denaturation or proteolytic cleavages resulted in enhanced binding by αXβ2 (Vorup-Jensen et al., 2005). It is possible that residues K^{242} and K^{243} on the αX I-domain may interact with the acidic residues of these decayed proteins.

The binding moieties of ICAM-1 for the αX I-domain were also evaluated in this study. In Figure 8B, the provided crystal structure shows domain 3 of ICAM-1 with two moieties recognized by the αX I-domain, D3a (D^{229}QRLNPTV) and D3c (E^{254}DEGTQRL). According to a structural study of ICAM-1, these moieties are located on the top surface of dimerized ICAM-1 molecules, allowing for easy access to its ligands (Yang et al., 2004). Integrin αMβ2 also binds to these moieties with the sequence D^{229}QR and E^{254}DE, thus suggesting a simi lar ionic interaction between these integrin I-domains and ICAM-1.

The mutation from EDE to KEK in domain 3 of ICAM-1 in duced only a 3-fold reduction in the binding affinity (Table 2), which appeared to be rather low, considering that EDE residues were found to be critically important for the αX I-domain.

This low level of reduction can be explained as follows: since the other regions of domain 3, such as the D^{229}QRLNPTV region, are also involved in the recognition of the αX I-domain, one mutation (EDE to KEK) may not prove sufficient to block binding to the αX I-domain. Additionally, one acidic residue (E) in KEK is capable of interacting with the basic residues of the αX I-domain in the form of ionic interactions, and binding activity thus remains at a low level. This is why mutation from EDE to KEK did not result in the abolition of the binding activity of the domain 3. However, this data still supports the notion that the region of domain 3 (E^{254}DEG) is important with regard to the αX I-domain.

Flick et al. reported that domain 4 of ICAM-1 was responsible for αXβ2 (Flick et al., 2005). Unexpectedly, we identified domain 3 of ICAM-1 as a primary binding site for the αX I-domain. Further investigations will be required to resolve this discrepancy, but two points must clearly be addressed. Our conclusions were derived from three lines of evidence: (1) SPR and solid phase analyses demonstrated that domain 3 of ICAM-1 (D3-Fc) binds to the αX I-domain; (2) the domain 3-specific peptides could bind to the αX I-domain, and inhibited the binding of domain 3 to the αX I-domain; (3) the domain 3 of ICAM-1 produced by *Pichia* showed significant binding affinity to the αX I-domain. Additionally, it was demonstrated that αMβ2 recognized domain 3 of ICAM-1. Considering the fact that αM and αX are closely related subunits with an amino acid identity of 63%, sharing biological functions, structure, and ligands (Stacker and Springer, 1991), it remains possible that αXβ2 can recognize domain 3 of ICAM-1 as αMβ2.

The second point to be addressed involves the results of two different experimental approaches: purified αXβ2 from leukocytes were employed by Flick et al., whereas recombinant αX I-domain was utilized in this study. Because cell surface αXβ2 molecules remain inactive until the leukocytes are stimulated, αXβ2 purified directly from unstimulated leukocytes may be inactive (Zang and Springer, 2001). This inactive integrin may differ from the I-domain in that it can recognize ICAM-1 domains.

Since the I-domain is the primary ligand-binding site of the β2 integrin, it is probable that integrin αXβ2 also binds to domain 3 of ICAM-1. Although the expression level of αXβ2 is lower than those of other β2 integrins, αXβ2 performs a significant role in leukocyte trafficking via interaction with the endothelium. The results of a previous study in which blocking antibodies were employed suggested a prominent role of αXβ2 in peripheral blood monocytes in adhesion to endothelial cell monolayers (te Velde et al., 1987). It was also reported that leukocyte infiltration was greatly reduced in the central nervous systems of αX-deficient mice immunized for experimental autoimmune encephalomyelitis (Bullard et al., 2007). Therefore, the results of this study may provide useful information for the development of potential treatments for chronic inflammatory diseases in which uncontrolled leukocyte infiltration occurs.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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