Characterization of αX I-domain binding to Thy-1

Jeongsuk Choi [a], Lisette Leyton [b], Sang-Uk Nham [a,*]

[a] *Divisions of Science Education and Biology, Kangwon National University, Choonchun, Kangwon, Republic of Korea*
[b] *FONDAP Center for Molecular Studies of the Cell, ICBM-Faculty of Medicine, University of Chile, Santiago, Chile*

Abstract

The β2 integrins are found exclusively in leukocytes and they are composed of a common β chain, CD18, and one of four unique α chains, CD11a (αL subunit), CD11b (αM subunit), CD11c (αX subunit), or CD11d (αD subunit). αX–β2 which binds several ligands including fibrinogen and iC3b is expressed in monocytes/macrophages and dendritic cells playing an important role in the host defense. Despite the unique characteristics on expression and regulation, αX–β2 is less functionally characterized than other β2 integrins. To understand the biological function of αX–β2 more, we tested the possibility that αX–β2 binds Thy-1, a membrane protein involved in cell adhesion and signaling regulation in neurons and T cells. Here we report that a ligand binding moiety of αX–β2, the I-domain, bound Thy-1 in a specific and divalent cation-dependent manner. The dissociation constant (K_D) of αX I-domain binding to Thy-1 was 1.16 μM and the affinity of the binding was roughly 2-fold higher than that of αM I-domain. Amino acid substitutions on the βD–α5 of αX I-domain (D249, KE243/244) showed low affinities for Thy-1 while other point mutations on α3–α4 and βE–α6 loops of I-domain did not, suggesting that Thy-1 recognizes the portion of a βD–α5 loop, possibly α5 helix. Taken together, these results indicate that αX–β2 specifically interacts with Thy-1. Additionally, kinetic analysis reveals a moderate affinity interaction in the presence of divalent cations. Given the reported role of Thy-1 in the regulation of T cell homeostasis and proliferation, it is tempting to speculate that αX–β2 may be involved in Thy-1 function.

Keywords: I-domain; Thy-1; Integrin; Leukocytes; Binding; αX–β2

Integrins are heterodimeric transmembrane proteins, providing a link between extracellular matrix and intracellular cytoskeleton [1]. The β2 (CD18) integrins, which share common β subunit (CD18), are expressed mainly on leukocytes: αL–β2 (LFA-1, CD11a/CD18), αM–β2 (Mac-1,CD11b/CD18), αX–β2 (p150,95, CD11c/CD18), and αD–β2 (CD11d/CD18). These integrins mediate several leukocyte activities such as adhesion, spreading, chemotaxis, migration, and phagocytosis, playing important roles in the host defense [2]. A variety of counter-receptors and soluble ligands for β2 integrins have already been described. In particular, αX–β2 has been shown to bind collagen type 1, iC3b, fibrinogen, LPS, and ICAM-1 [3–5]. αX–β2 has many biological functions and molecular structure similar to those of αM–β2. However, αX–β2 has a unique cellular expression on monocytes, macrophages, and $CD8^-$ dendritic cells. [6]. Additionally, the regulation and activation processes of this integrin seem to be quite different from those of αM–β2 [7].

Several types of α integrin subunits (αL, αM, αX, αD, αE, α1, α2, α10, and α11) possess characteristic inserted domains (I-domains), which consist of about 200 amino acids. I-domains are the site for ligand binding and have a unique structure with 6–7 α helixes and 6 β sheets to form an independent structural and functional unit [8]. A metal ion dependent adhesion site (MIDAS) has been identified to locate in the upper surface of the I domain where ligand binding occurs. There are five exposed loops surrounding MIDAS, which undergo conformational changes to bind their physiological ligands [9].

* Corresponding author. Fax: +82 33 251 7091.
E-mail address: sunham@cc.kangwon.ac.kr (S.-U. Nham).

In mutation analysis of αX and αM I-domains, the loops close to metal binding sites are important portions for ligand recognition. Point mutations on the βD–α5 loop of αM I-domain abolish the binding of iC3b [10]. A study with homolog-scanning mutagenesis on the αM I-domain suggested that the βA–α1, α3–α4, and βD–α5 loops are responsible for iC3b binding [11] and βD–α5 loop for fibrinogen association [12,13] . In αX I-domain, the α3–α4 and βD–α5 loops are important for the recognition of fibrinogen [14].

Thy-1 (CD90) is a glycosyl phosphatidylinositol (GPI)-anchored glycoprotein member of the immunoglobulin superfamily [15,16]. Thy-1 is expressed in various cells including neuronal cells, thymocytes, fibroblasts, and activated endothelial cells [17,18]. The function of Thy-1 seems to be related to cell adhesion and activation. Several reports show that Thy-1 promotes the adhesion of thymocytes to thymic epithelia and astrocytes to neurons, as well as activation of T cells [19–21]. Recently, it has been described that human Thy-1 specifically bound to αM–β2 (CD11b/CD18, Mac-1) on the leukocytes, and that this binding led to adhesion of leukocyte to activated endothelial cells (Thy-1^+) and subsequent transendothelial migration [22].

Considering the unique characteristics of αX–β2 expression and regulation, the biology of αX–β2 may be important to understand the defense mechanisms of our body. However, αX–β2 is less functionally characterized compared to the other β2 integrins. Here, a series of experiments was performed to identify the unknown αX–β2 ligands in an attempt to analyze its function further. Recombinant αX I-domain was employed to demonstrate and characterize its interaction with Thy-1 molecule. In addition, the structural and functional relationship of αX I-domain responsible for the binding to Thy-1 was investigated.

Materials and methods

Purification of αM and αX I-domains. The αX I-domain was purified as reported previously [23]. Briefly, *Escherichia coli* (JM109) was transformed with the constructed plasmid (pEX-CD11cI) and then induced to express the αX I-domain fused with glutathione *S*-transferase (GST-αX I-domain) by isopropyl-thio-β-D-galactoside (IPTG). αM I-domain cDNA was sub-cloned by polymerase chain reaction from phCD11b containing αM cDNA. Polymerase chain reaction (PCR) primers were synthesized to generate a translational stop codon and a restriction enzyme site (*Bam*HI). The DNA fragment was sub-cloned in a cloning vector pGEM5fz (Promega, USA), and sequenced by a dideoxy chain termination method. The DNA was digested with *Bam*HI and *Xho*I, and sub-cloned in the *Bam*HI and *Xho*I sites of pGEX-5T-3 vector (Pharmacia Biotech., USA) to form an expression plasmid (pEX-CD11b I).

Bacterial cells [Bl-21(DE3)] transformed by pEXCD11bI or pEXCD11cI were incubated to a log phase of growth ($A_{600} = 0.3$–0.5) and then treated with 0.5 mM IPTG for 4 h. Resuspended cells in 10 ml of 20 mM Tris/HCl, pH 7.5, 10 mM ethylenediaminetetraacetic acid disodium (EDTA) after centrifugation were lysed by using a high pressure homogenizer (French press). Bacterial cell lysates were loaded onto a glutathione–Sepharose 4B column and the fusion protein was eluted by 10 mM reduced glutathione in 20 mM Tris/HCl, pH 8.0. The purified fusion protein was dialyzed against Hepes-buffered saline (HBS, pH 7.4) after assessment of homogeneity of the eluted protein by SDS–polyacrylamide gel electrophoresis.

Site-directed mutagenesis. Site-directed mutagenesis was carried out by using a Quickchange mutagenesis kit (Stratagene, USA). The mutated positions of the I-domain, substituted amino acids, and used mutagenic primers are shown in Table 1. The amino acids to be substituted for alanine were chosen from a previous mutagenesis study of αM I-domain, in which mutations might affect ligand binding but do not cause a drastic change on the conformation of the I-domain [10]. The other amino acids were changed into the one from an α2 sequence, because α2 possesses a similar three dimensional structure to αX I-domain and does not bind to Thy-1 [9]. Polymerase chain reaction (PCR) was carried out with pSPCD11cI (1 ng) as a template, *Pfu*I (2.5 U), and a set of primers (2 μM) in a 50 μl reaction mixture. The amplified cDNA (1 μl) was treated with *Dpn*I (10 U) at 37 °C for 1 h to remove parental plasmid and transform *E. coli* (XL-1 blue). Plasmids were isolated from the transformants and all constructs were verified by DNA sequencing. Mutants were cloned into pGEM5fz to form expression plasmids and then introduced into *E. coli* for the expression and purification of the mutant fusion proteins.

Purification of Thy-1. Recombinant Thy-1 fused with Fc (Thy-1-Fc) was purified as described in Leyton et al. [20]. Briefly, Thy-1-Fc expression vector containing a cDNA encoding amino acid 1–131 of Thy-1 and a Fc fragment of human IgG1 was introduced into HEK-293 cells. Supernatants from stably transfected cells were harvested and subjected to a protein A affinity chromatography. The purity of isolated proteins was assessed by a SDS–PAGE analysis under non-reducing conditions.

Characterization of binding by surface plasmon resonance (SPR) analysis. Binding analysis was carried out in a Biacore X (Biacore, Sweden). Thy-1-Fc or BSA as control was covalently immobilized to carboxymethyl dextran surface of CM5 chip via primary amino groups,

Table 1
Nucleotide sequences of the primers used in this study

Mutants[a]	Mutagenic primer[b]
S199A	GCCTGTTGGCTgCTGTTCACCAGCTG
Q202A	GCTTCTGTTCACgcGCTGCTGCAAGGGTTTAC
LQ203/204GG	CTTCTGTTCACCAGgggggaGGGTTTACATACACG
KE243/244SH	ATCACTGATGGGAAGAgccatGGCGACAGCCTGGAT
D249M	GAAGGCGACAGCCTGatgTAcAAGGATGTCATCCCC
Y250A	GACAGCCTGGATgcTAAGGATGTCATCCC
Q274A	GATTAGCTTTTgcAAACAGAAATTCT

[a] In the name of mutants, the first letter indicates the amino acid residue at the position of CD11c 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.
[b] The lowercase letters indicate the mutagenic bases.

using an amine coupling kit. Analytes, GST-αX I-domain, αX I-domain mutants, and GST-αM I-domain, were flowed over the sensor chip at 30 μl/min, 25 °C. HBS with 1 mM $MgCl_2$ was used as a running buffer, and 20 mM Tris (pH 8.0), 0.3 M NaCl, and 20 mM EDTA were used to remove bound protein and regenerate the surface of the sensor chip. Binding curves were obtained by subtracting bulk change and non-specific binding. Kinetics was analyzed by Biaevaluation 3.0 software. K_D, k_{on}, and k_{off} were calculated by curve fitting of association and dissociation phases using a 1:1 Langmuir binding model.

Results

Since I-domains of β2 integrins are primary binding sites for their ligands, we used αX I-domain to test if αX–β2 is able to interact with Thy-1. A previous study employing purified αM–β2 indicated that the addition of excess amount of I-domain was able to inhibit the interaction of Thy-1 with αM–β2 [22]. To begin the test, αX I-domain and αM I-domain with GST were expressed in *E. coli* and purified. Human and mouse Thy-1-Fc (hThy-1-Fc and mThy-1-Fc, respectively) were expressed in HEK293 cells and purified as previously described. As shown in Fig. 1, the purified GST-I-domains and purified GST migrated as major bands of 48 and 26 kDa, respectively. These sizes are consistent with their predicted molecular weights. The purified human or mouse Thy-1-Fc migrated as single bands of 106 kDa, equivalent to the dimeric form of the molecule as reported [20]. The other mutant I-domains showed the same molecular weights and homogeneity in a SDS–PAGE analysis as the αX I-domain shown in Fig. 1 (data not shown).

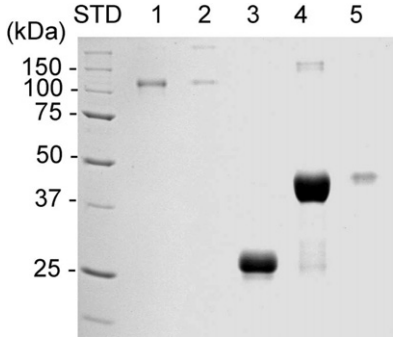

Fig. 1. SDS–PAGE analysis of purified proteins. αX I-domain and αM I-domain, expressed as GST fusion proteins, were purified on a glutathione–Sepharose column. Human and mouse Thy-1, expressed as Fc fusion proteins, ware purified on a protein A column. All proteins were subjected to 10% SDS–PAGE in a non-reducing condition and stained with Coomassie blue. Lane 1, human Thy-1-Fc; lane 2, mouse Thy-1-Fc; lane 3, GST; lane 4, GST-αX I-domain; and lane 5, GST-αM I-domain.

To test whether αX I-domain binds Thy-1, various concentrations of GST-αX I domain were injected to let it interact with immobilized hThy-1-Fc. Very little binding was observed in the presence of EDTA or GST only, suggesting a specific binding (Fig. 2A). Fig. 2B shows that binding of I-domain to hThy-1-Fc is dose-dependent and saturable. Previous studies for the effects of cations on ligand binding to I-domains showed different results ranging from dependence to complete independence [24,25]. To determine the cation dependence of αX I-domain binding to hThy-1-Fc, SPR analysis was carried out in the presence of 1 mM each of Ca^{2+}, Mg^{2+}, and Mn^{2+}. Fig. 3 indicates that αX I-domain requires divalent cations for hThy-1-Fc binding. αX I-domain binds hThy-1-Fc at a high level in the presence of Mg^{2+} and Ca^{2+}.

In the presence of Ca^{2+}, however, αX I-domain seems to dissociate from hThy-1-Fc faster than in Mg^{2+}, resulting in a low level of affinity. The binding level of αX I-domain with hThy-1-Fc in Mn^{2+} ion is lower than that in Mg^{2+}.

The binding kinetics of αX I-domain and αM I-domain were analyzed and compared with each other. Kinetic constants were measured from the association and dissociation phases of each protein binding. K_D was calculated from observed k_{off}/k_{on} (Table 2). The dissociation constant (K_D) of αX I-domain binding to hThy-1-Fc was 1.16 μM and the affinity of the binding was roughly 2-fold higher than that of αM I-domain. The high binding affinity of αX I-domain was mainly caused by the k_{off} which is nearly half of that for αM I-domain. These

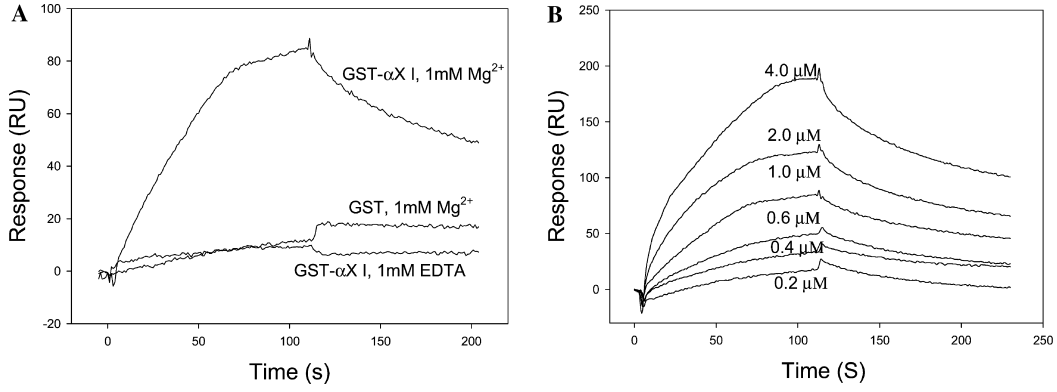

Fig. 2. SPR sensorgram of αX I-domain binding to immobilized hThy-1. Human Thy-1-Fc was immobilized on a CM5 sensor chip at 4000 RU, response unit. (A) One micromole of GST-αX I-domain or GST was injected to the immobilized human Thy-1 in the presence of 1 mM Mg^{2+} or EDTA. (B) Different concentrations of αX I-domain, ranging from 0.2 to 4 μM, were injected to the Thy-1.

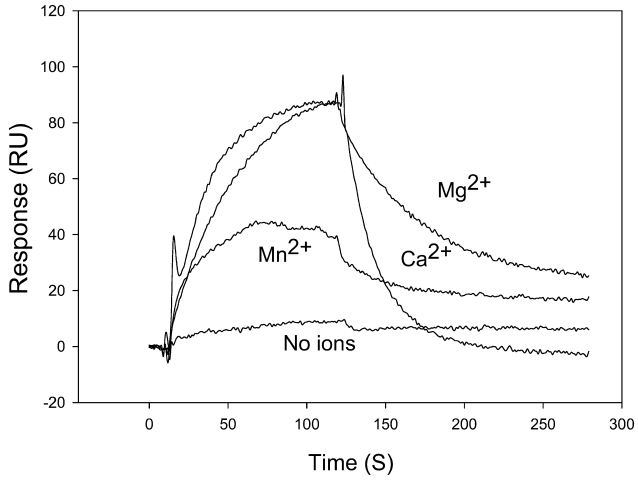

Fig. 3. SPR sensorgram of αX I-domain binding to Thy-1 in the presence of Ca^{2+} Mg^{2+}, and Mn^{2+}. One micromole of αX I-domain, with various cations at the final concentration of 1 mM, flowed over an immobilized human Thy-1 (4000 RU).

Table 2
Kinetics of αX I-domain and αM I-domain binding to Thy-1

Analyte	Ligand	K_D (10^{-6} M)	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})
CD11cI	hThy-1	1.16 ± 0.14	6760 ± 1700	0.0072 ± 0.0012
CD11bI	hThy-1	2.40 ± 0.46	6070 ± 1500	0.0142 ± 0.0008
CD11cI	mThy-1	1.07 ± 0.17	6130 ± 790	0.0065 ± 0.00018

data suggest that αX–β2 associates, hThy-1 at the same level but dissociates a little slower than αM–β2. The binding affinity of human and mouse Thy-1 to αX I-domain appears to be at the same level, indicating that αX I-domain binds equally well to both human and mouse Thy-1. These data suggest that there is no species specificity in the binding of αX–β2 to Thy-1.

As a next step, we further characterized the nature of the interaction by identifying some of the amino acid residues on αX I-domain involved in the Thy-1 binding. Several residues were chosen based on assumptions of the previous studies, showing that two flexible loops such as α3–α4 and βD–α5 played important roles in ligand recognition in the αX I-domain and αM I-domain [10–14]. The amino acids with charged or polar side chain were chosen for point mutations to be replaced by alanine or the corresponding amino acids in an α2 I-domain sequence. The binding ability of each protein was expressed as dissociation constants (K_D) and is shown in Table 3. D249M mutant protein revealed the lowest affinity among the αX I-domain mutants. The binding affinity of D249M mutant protein was one-eighth of wild type and that of KE243/244SH mutant protein was one-third. These mutations located on the βD–α5 loop of I-domain are very close to α5 helix, suggesting that Thy-1 recognizes a portion of βD–α5 loop, possibly the α5 helix as well. This observation agrees with a previous report showing the ligand interacting moiety in αM I-domain [13].

Table 3
The binding kinetics of αX I-domain mutants

Mutants	Loop	K_D (10^{-6} M)
Wile type		1.16 ± 0.14
S199A	α3–α4	1.18 ± 0.50
Q202A	α3–α4	0.93 ± 0.26
LQ203/204GG	α3–α4	1.09 ± 0.24
KE243/244SH	βD–α5	3.46 ± 0.81
D249M	βD–α5	8.23 ± 1.22
Y250A	βD–α5	0.99 ± 0.08
Q274A	βE–α6	1.75 ± 0.89

Discussion

Although the Thy-1 was identified several decades ago, its biological role remains unclear. One of the problems related to the biology of Thy-1 is that ligands or counter-receptors for Thy-1 are just beginning to emerge. Thus far integrin αV–β3, αM–β2, and Thy-1 itself have been identified as ligands/counter-ligands of Thy-1 [18,20,22].

In this study, we demonstrate that αX–β2 is also a ligand for Thy-1. The binding of Thy-1 to the I-domain, a ligand binding moiety of αX–β2, was specific and required divalent cations such as Mg^{2+}, Mn^{2+}, and Ca^{2+}. This finding further suggests that the αX–β2 and Thy-1 interaction may mediate leukocyte extravascular migration during inflammation, because Thy-1 is expressed on endothelial cells when these are activated in inflamed tissues [18]. Although the expression level of αX–β2 on leukocytes is not as high as αM–β2 at the initial stages of transendothelial migration, it is likely that αX–β2 plays a role as important as αM–β2.

αX–β2 is highly expressed on dendritic cells, being recognized as a dendritic cell marker. Dendritic cells are potent antigen presenting cells (APC) that regulate the immune response through the induction of adaptive immunity [26]. It is shown that Thy-1 is expressed on thymocytes and peripheral T cells in mice. Several lines of evidence indicate that the binding of Thy-1 to putative ligand on dendritic cells together with CD28-CD80/CD86 interaction induces signals to trigger T cell proliferation and interleukin 12 production [27]. Data presented in this study suggest that as a major surface molecule on dendritic cells, αX–β2 may be involved in the regulation of T cell homeostasis through T cell proliferation and cytokine production via its interaction with Thy-1. However, further studies are required to probe this possibility.

Most of integrin I-domains require Mg^{2+} and Mn^{2+}, but not Ca^{2+}, for the ligand binding. Mg^{2+} and Mn^{2+} support I-domain binding to the ligands and Ca^{2+} actually inhibits Mg^{2+}-mediated binding [28]. However, some integrins such as α1 I-domain bind collagen and laminin in Ca^{2+} as well as in Mg^{2+} or Mn^{2+} [29]. The stimulatory effect of Ca^{2+} on the ligand binding was also observed in this study. The reason for the opposing effects of Ca^{2+} is not well known. A recent report suggests that cation binding affinity and subsequently ligand binding are regulated

by both activation state of I-domain as well as the ligand itself [30]. Thus, it will be interesting to explore the possibility that Thy-1 itself may regulate αX I-domain binding to both the metal ion and Thy-1.

A previous study revealed that two loops of αX I-domain such as α3–α4, βD–α5 were critical for the recognition of fibrinogen [14]. In αM–β2, the βD–α5 loop and the α5 helix segment (K245–D261) are critical portions in recognizing several ligands including fibrinogen, fibronectin, and ovalbumin. [13]. The data presented here are in agreement with previous results, strengthening the notion that the βD–α5 loop is an important moiety in β2 integrins for recognition of their ligands. Results presented also suggest that the α5 helix segment of αX I-domain plays a significant role in this interaction. This possibility and the critical residue for Thy-1 recognition are at present under investigation.

Acknowledgments

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