Rational design of a novel calcium-binding site adjacent to the ligand-binding site on CD2 increases its CD48 affinity

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Abstract

Electrostatic interactions are important for molecular recognition processes including Ca^{2+} -binding and cell adhesion. To understand these processes, we have successfully introduced a novel Ca^{2+} -binding site into the non- Ca^{2+} -dependent cell adhesion protein CD2 using our criteria that are specifically tailored to the structural and functional properties of the protein environment and charged adhesion surface. This designed site with ligand residues exclusively from the β -sheets selectively binds to Ca^{2+} and Ln^{3+} over other mono- and divalent cations. While Ca^{2+} and Ln^{3+} binding specifically alters the local environment of the designed Ca^{2+} -binding site, the designed protein undergoes a significantly smaller conformation change compared with those observed in naturally occurring Ca^{2+} -binding sites that are composed of at least part of the flexible loop and helical regions. In addition, the CD2–CD48-binding affinity increased approximately threefold after protein engineering, suggesting that the cell adhesion of CD2 can be modulated by altering the local electrostatic environment. The study provides site-specific information for regulating cell adhesion within CD2 and gives insight into the structural factors required for Ca^{2+} -modulated biological processes.

Keywords: calcium-binding sites; cell adhesion; metal-binding affinity; metal selectivity; rational design

Cell adhesion molecules are a large group of proteins involved in diverse cell-to-cell and cell-to-extracellular matrix interactions. Ca^{2+} -dependent cell adhesion proteins such as cadherins form homophilic dimers from different cells, which are important for the development of multicellular organisms, and a prerequisite for maintaining tissue integrity by stabilizing cell-to-cell contacts (Herrenknecht 1996). Non- Ca^{2+} -dependent cell adhesion proteins, such as CD2, which is expressed on most human T cells and natural killer (NK) cells, initiate and maintain contact with antigen-presenting or target cells and play an

important role in signaling (Driscoll et al. 1991; van der Merwe et al. 1995; Blattman and Greenberg 2004). The membrane-distal immunoglobulin-like (Ig-like) domain of CD2 (domain 1) is responsible for molecular recognition and has a similar topology to that of the Ca^{2+} -dependent cell adhesion molecule cadherin. In contrast to strong and focused enzyme/inhibitor interactions, a relatively large surface area is buried in the weak CD2 protein–protein interactions (Nooren and Thornton 2003). In addition to their low binding affinities ($K_d > \mu\text{M}$), the highly dynamic, transient, and reversible nature of CD2 interactions (van der Merwe and Barclay 1994; Chen et al. 2000) make understanding the key factors that contribute to the molecular recognition challenging.

To date, CD2 is one of the most extensively studied cell adhesion proteins and has served as a model system to

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identify the key determinants involved in molecular recognition. CD2 consists of two extracellular Ig-like domains and an intracellular C-terminal region linked by a single transmembrane region. The physiological binding partner of CD2 is CD48 in rodents and CD58 in humans. Heterophilic protein–protein interaction at the cell surface has been identified using mutagenesis and structural studies (Selvaraj et al. 1987). The GFCC'C" surface, formed by the β -strands G, F, C, C', and C'', is relatively flat and highly charged for both rat and human CD2. Approximately 35% and 70%, respectively, of the surface residues in the rat and human CD2 ligand-binding site are charged, compared with 19% for the average solvent-exposed protein surface (Chen et al. 2000). Seven charged residues (D28, E29, R31, E33, E41, K43, and R87) and three aromatic or hydrophobic residues (Y81, F49, and L38) located on the GFCC'C" surface contribute to the recognition of CD48 (Davis et al. 1998). Mutations with reversed surface charges have been shown to result in weak binding or loss of binding to CD48 (van der Merwe et al. 1995). The ligand-binding ability is restored if there is a complementary charge reversal on the CD48 surface; this suggests the importance of the electrostatic complementarity for the binding interaction (Davis et al. 1998).

Electrostatic interactions are also important for Ca^{2+} -binding. Ca^{2+} is predominantly chelated by oxygen atoms from side chains of Asp, Glu, Asn, main chain carbonyl, and solvent (Yang et al. 2002). A high percentage of charged ligand residues have been observed in naturally occurring Ca^{2+} -binding sites (Marsden et al. 1990), and clustering of charged ligand residues contributes to Ca^{2+} -binding affinity. In addition, analyses of the structural characteristics of over 500 Ca^{2+} -binding sites in proteins have shown that these sites often lie within loops on or near the protein surface. The relative population of protein-derived Ca^{2+} ligand residues are 72.5%, 13.9%, and 13.5% within the turn/loop, helix, and sheet secondary structure, respectively (Strynadka and James 1993; Pidcock and Moore 2001). For example, several Ca^{2+} -binding sites are clustered together at the flexible linker regions and are formed by ligand residues from different portions of the protein. Furthermore, to be essential for cell adhesion, calcium binding also increases protein stability and induces oligomerization (Koch et al. 1997; Prasad and Pedigo 2005). To date, to our knowledge there have been no reports of a naturally occurring Ca^{2+} site that possess ligand residues all from the β -sheets.

Protein design is an important tool that allows for the study of the structural and functional properties of proteins. The design of Ca^{2+} -binding proteins provides insight into the key determinants for Ca^{2+} -binding without the difficulties of metal–metal interactions observed in proteins with multiple Ca^{2+} -binding sites (Wilkins et al. 2003). We have previously reported our success in

the de novo design of Ca^{2+} -binding sites in non- Ca^{2+} -binding proteins without splicing in an entire known metal-binding domain (Yang et al. 2003, 2005b). The designed protein Ca.CD2 was shown to bind Ca^{2+} at the intended site with the designed ligand arrangement resulting in good metal selectivity.

In this paper, we report our success in engineering a novel Ca^{2+} -binding site into CD2 with all the potential ligand residues from the β -sheet regions of the protein to understand electrostatic interactions, in particular, in molecular interaction and Ca^{2+} -binding. Using various spectroscopic methods, we have shown that this protein with the designed Ca^{2+} -binding site selectively binds Ca^{2+} over other mono- and divalent cations. While Ca^{2+} and Ln^{3+} binding specifically alter the local environment at the designed Ca^{2+} -binding site, NMR studies reveal that the designed protein undergoes a significantly smaller conformation change compared with those observed in naturally occurring Ca^{2+} -binding sites that are comprised of at least part of the flexible loop and helical regions. Interestingly, the introduction of negatively charged Ca^{2+} ligands increased the CD2–CD48-binding affinity, suggesting that the molecular recognition capability of CD2 can be modulated by altering the local charge environment.

Results

Rational design and selection of the novel Ca^{2+} -binding site

This study endeavors, using protein design and structural analysis, to achieve our objective of elucidating the role of electrostatic interactions on Ca^{2+} -binding affinity and molecular recognition. We have designed a potential Ca^{2+} -binding site within the backbone of domain 1 of CD2 (1hng). In addition to the use of the pentagonal–bipyramidal geometry and a library of side chain rotamers of ligand residues (or atoms from the main chain) with strong Ca^{2+} -binding preferences (Davis et al. 1998; Wilkins et al. 2002; Yang et al. 2003), several new criteria were applied for the design and selection processes. First, we introduced the bidentate ligand Asp (Pidcock and Moore 2001) since it has a strong β -sheet propensity, which is important for stabilizing the native conformation. Second, the site was engineered adjacent to the CD48-binding site in order to modulate the adhesion. Third, residues known to be essential for CD48 binding were not mutated (Fig. 1A). Fourth, solvent accessibility, side chain steric conflicts with existing atoms, disruption of hydrogen bonding, conserved folding residues, and hydrophobic interactions (Yang et al. 2005b) were also considered.

Figure 1A shows the model structure of the selected Ca^{2+} -binding site CD2.6D79 potentially formed by ligand residues all from the β -sheet regions of the host protein.

Figure 1. Structural properties of CD2.6D79. (A) The model structure of the designed Ca^{2+} -binding site (CD2.6D79) in domain 1 of rat CD2 based on 1hng (Chen et al. 1998) generated by PyMOL (DeLano Scientific). (Red) Ca^{2+} coordination residues N77, D79, N90, and D92, (blue highlight) essential residues involved in the recognition of CD48. Residue R87 is in ball-and-stick representation (black). (B) Surface potentials of the CD48-binding surface, displayed using GRASP (Nicholls et al. 1991), of CD2 (left), CD2.R87A (middle), and CD2.6D79 (right) show potential difference at residue 87. (Red) Negative potentials, (blue) positive potentials. (C) Electrostatic potential of residue 87 in the proteins CD2, CD2.R87A, CD2.6D79, CD2.T79D, and CD2.A92E revealing the negative potential of R87 in CD2.6D79 and CD2.R87A, which accounts for increased charge complementarity of the surface to CD48.

The proposed Ca^{2+} -binding site of this mutant has a coordination number of six with D79 as its bidentate ligand, hence the name CD2.6D79. The proposed Ca^{2+} -binding pocket is formed by the side chain carboxyl oxygen atoms of two charge mutations, T79D and A92E, and two natural amino acids, N77 and N90. Residues D79 and N77 are from the F β -strand, while E92 and N90 are from the G β -strand of the protein (Fig. 1A). Site CD2.6D79 is adjacent to the essential cell adhesion residues E33, R31, and Y81. Figure 1B shows the electrostatic surface of CD2, the CD2 variant with a single mutation of R87A, and CD2.6D79. Electrostatic calculations show that the surface potential of CD2.6D79 compared with CD2 decreased by 74.1 kcal/mol. The mutation T79D contributes 1.8-fold greater (63%) to the perturbation of the electrostatic surface than A92E (37%).

CD2.6D79 retains its native structure but has a reduced thermal stability

Several spectroscopic techniques were employed to analyze the secondary and tertiary structure of CD2.6D79. The far-UV CD and Trp fluorescence spectra of CD2.6D79 are almost identical to that of wild-type CD2 (Fig. 2A,B). CD2.6D79 exhibits similar binding affinities

to the conformation-sensitive antibodies OX34 and OX55 as CD2 (data not shown), which further suggests that the introduction of the Ca^{2+} -binding site does not alter the secondary and tertiary structure of CD2.

No detectable change in the CD and fluorescence spectra of CD2.6D79 was observed upon the addition of Ca^{2+} (Fig. 2A,B). High-resolution NMR was then utilized to reveal the detailed structural change of CD2.6D79. The chemical shift changes between the apo-form (1 mM EGTA) and the loaded form (10 mM Ca^{2+}) for the majority of the residues at the β -strands including those from the residues around the Ca^{2+} -binding site such as V78, Y81, and K91 are very small (<0.05 ppm). In contrast, residues such as E41, K43, and K45 at the flexible loop regions exhibit more pronounced changes, similar to that of CD2 (Chen et al. 2002). Since various studies have shown that the residues from the β -strands F and G are significantly more rigid than those from the loops of the protein (Driscoll et al. 1991; Yang et al. 2005a), the lack of overall perturbation in the chemical shifts as a consequence of metal binding indicates a limited conformational change in the Ca^{2+} -binding site formed by a less dynamic secondary structure (β -strand).

We have further investigated the thermal denaturation of CD2.6D79. The CD and fluorescence spectra of

Figure 2. Conformational analyses of CD2.6D79. (A) The far-UV CD spectra of CD2.6D79 in the absence (triangles) and presence (squares) of 10 mM Ca²⁺ with the spectrum of CD2 (circles). (B) The Trp fluorescence spectra of CD2.6D79 in the absence (triangles) and presence (squares) of 10 mM Ca²⁺ and the spectrum of CD2 (circles). (C) Thermal transitions in the absence (squares) and presence of Ca²⁺ (circles) give T_m values of 49°C ± 1°C and 53°C ± 1°C, respectively. (D) The relative binding of CD2.6D79 and CD2 to CD48 in the absence of metal (1 mM EGTA, white bars) as well as in the presence of 100 μM La³⁺ (dark gray bars) or 10 mM Ca²⁺ (light gray bars).

CD2.6D79 at 25°C and 90°C are very similar to that of wild-type CD2 (data not shown), suggesting that both proteins have similar native and thermal denaturation states. The CD signals at 218 and 222 nm were used to monitor the thermal transition curves of CD2.6D79 in the presence and absence of Ca²⁺, which are consistent with a two-state model (Fig. 2C) and are reversible, as is wild-type CD2. The measured T_m values for the Ca²⁺-free and Ca²⁺-bound forms of the protein are 49°C ± 1°C and 53°C ± 1°C, respectively, while that for CD2 is 62°C. This decrease in thermal stability is likely due to electrostatic repulsion effects.

CD2.6D79 binds to Ca²⁺ and Ln³⁺ with metal selectivity

Ln³⁺ ions have similar ionic radii and metal coordination chemistry as Ca²⁺ and are commonly used to probe Ca²⁺-binding (Horrocks 1993; Drake et al. 1997) due to the

silent spectroscopic properties of Ca²⁺, especially when the protein lacks conformational change. Direct binding of Ln³⁺ was investigated using FRET between aromatic residues and Tb³⁺. As shown in Figure 3A, when excited at 280 nm, fluorescence at 545 nm gradually increases with the addition of Tb³⁺, giving a K_d of 15.3 ± 1.5 μM to CD2.6D79 (Fig. 3B; Table 1). In addition, this Tb³⁺ fluorescence signal was significantly decreased upon the addition of 10 mM Ca²⁺ or 0.1 mM La³⁺ to the Tb³⁺-protein complex, suggesting that Ca²⁺ and La³⁺ competitively displaced the bound Tb³⁺ ion. The metal binding affinity of 7.4 ± 1.3 μM for La³⁺ was obtained using this competitive binding (Fig. 3B, inset; Table 1). The greater affinities for Tb³⁺ and La³⁺ over Ca²⁺ are likely due to the additional positive charge for these metal ions. In contrast, addition of 10 mM Mg²⁺, 10 mM K⁺, 0.1 mM Ni²⁺, and 0.1 mM Co²⁺ did not significantly decrease the fluorescence intensity, indicating that these concentrations of

Figure 3. Metal binding of CD2.6D79. (A) Enhancement of fluorescence signal at 545 nm as Tb^{3+} concentration is increased (from bottom to top: 0, 2.0, 6.0, 11.9, 26.3, and 124.3 μM). (B) Fractional change of fluorescence enhancement at 545 nm as a function of Tb^{3+} . (Inset) Fluorescence signal decreases at 545 nm as a function of La^{3+} . (C) Tb^{3+} fluorescence decreases in the presence of Ca^{2+} (10 mM), La^{3+} (100 μM), Mn^{2+} (100 μM), Mg^{2+} (10 mM), K^{+} (10 mM), Ni^{2+} (100 μM), and Co^{2+} (100 μM). (D) 1D ^1H -NMR of the amide region of CD2.6D79 in the absence and presence of 600 μM Ca^{2+} or 600 μM La^{3+} . (E) Amide proton chemical shift differences of the La^{3+} -bound and La^{3+} -free forms of CD2.6D79. (F) The changes in the ^1H - ^{15}N HSQC spectra upon the addition of Ca^{2+} . (Black) Ca^{2+} -free, (red) Ca^{2+} -bound.

metals ions cannot compete with Tb^{3+} (Fig. 3C). The limited solubility of Ni^{2+} , Co^{2+} , and Mn^{2+} under the experimental conditions prevents further quantitative competition study at higher metal concentrations, but it is noteworthy that the physiological concentrations of these free metal ions are also very low (Finney and O'Halloran 2003). The results show that CD2.6D79 exhibits selective binding of Ca^{2+} over K^{+} and Mg^{2+} and selective binding of Ln^{3+} over Ni^{2+} and Co^{2+} . Like other natural Ca^{2+} -binding proteins, the same concentration of the divalent metal ion Mn^{2+} is able to compete with Tb^{3+} for the binding pocket, as is the trivalent ion La^{3+} , although to a lesser extent (Mildvan and Cohn 1970; Ubach et al. 1998).

Although the majority of residues do not show a significant change in their chemical shifts, addition of Ca^{2+} to CD2.6D79 specifically results in a modest resonance change at 9.7 ppm in 1D ^1H NMR, which is due to the designed Ca^{2+} ligand residue Asn-77 (Fig. 3D).

Also, the addition of the Ca^{2+} analog La^{3+} results in a considerable resonance change of residue Asn-77 (Fig. 3D). In addition, significant chemical shift changes in the amide region corresponding to the residues in proximity, such as W32 (11.8 Å) and Y76 (11.6 Å), are observed with La^{3+} binding in the 1D spectra. Further, 2D total

Table 1. The metal binding affinity and thermal stability of CD2.6D79

Ions	K_d (μM)	T_m ($^{\circ}\text{C}$)
EGTA	N/A	49 ± 1
Ca^{2+}	630 ± 50^a	53 ± 1
Tb^{3+}	15.3 ± 1.5^b	58 ± 2
La^{3+}	7.4 ± 1.3^b	56 ± 1

^aNMR: 20 mM PIPES, 10 mM KCl pH 6.8.

^bFRET: 100 mM MOPS pH 6.8.

correlation spectroscopy (TOCSY) experiments reveal significant chemical shift changes in residues that are in close proximity to the metal-binding site (Table 2; Fig. 3E), including R31 (10 Å) and K91 (5 Å). This is consistent with the greater effect of La^{3+} than Ca^{2+} on the Ca^{2+} ligand residues, possibly due to the greater charge number of the La^{3+} ion and the increased metal binding affinity (Table 1; Horrocks 1993; Drake et al. 1997). To further analyze its Ca^{2+} -binding properties and the effect of Ca^{2+} -binding on the conformation of the protein, we have applied ^1H - ^{15}N heteronuclear single quantum correlation spectroscopy (HSQC). Figure 3F shows the HSQC spectra of 6D79 in the absence (1 mM EGTA) and presence of 10 mM Ca^{2+} . The most significant peak shifting following addition of Ca^{2+} occurred in the side chain protons of N77 (Fig. 3F). By monitoring this resonance change as a function of Ca^{2+} concentration, a dissociation constant for Ca^{2+} of 0.63 ± 0.05 mM was obtained, a value comparable to those of naturally occurring extracellular Ca^{2+} -binding proteins such as cadherin (Pokutta et al. 1994; Yang et al. 2000). These results suggest that Ca^{2+} and La^{3+} specifically interact with the designed Ca^{2+} ligand residues without imposing a significant conformational change on CD2.

We have applied a paramagnetic relaxation NMR method to further examine the location of metal binding. Mn^{2+} binds to the Ca^{2+} -binding sites in proteins and induces line-broadening effects on the neighboring residues. The Mn^{2+} ion displays strong relaxation effects on nuclei that are proportional to r^{-6} , where r is the distance between the proton and the Mn^{2+} ion, and therefore can be used to predict the location of bound metal ions (Ubach et al. 1998; Yang et al. 2005b). Figure 4, A and B, show the ^1H - ^{15}N HSQC spectra of CD2.6D79 with 1 mM Ca^{2+} in the absence and presence of 10 μM Mn^{2+} . The coexistence of Ca^{2+} reduces the nonspecific inter-

actions of Mn^{2+} . Thus, Mn^{2+} primarily binds to the specific location, in this case, the Ca^{2+} -binding position. Consistent with relatively large $\text{Ca}^{2+}/\text{La}^{3+}$ -induced chemical shift changes for the residues around the metal binding pocket, the strongest broadening effects at low concentrations of Mn^{2+} were observed in the backbone of the designed ligand residues N77, N90, E92, and D79. Extensive broadening was also observed in the side chains of N77 and N90. Additionally, broadening effects were also detected, but to a lesser extent, for residues around the metal-binding site, including E33, R31, and K91. The results demonstrate that both Mn^{2+} and Ca^{2+} -binding occur close to these residues. Since the resonance intensity decreases and the HN-Ca^{2+} distances calculated from the model structure (Fig. 4C) are consistent with the theoretical relationship, taken together, our studies support our conclusion that the metal ions bind to the designed metal-binding site.

CD2.6D79 binds CD48 with an enhanced affinity

The biological function of CD2.6D79 was examined using surface plasmon resonance (SPR) to measure the binding to its target molecule CD48 using previously reported methods (Davis et al. 1998; Yang et al. 2005b). As shown in Figure 2D, a threefold decrease in the dissociation constant for CD48 binding was observed for CD2.6D79 compared with wild-type CD2 in the absence of metal (1 mM EGTA). Addition of 10 mM Ca^{2+} or 0.10 mM La^{3+} did not significantly alter the affinity of CD48 binding to CD2.6D79. Our results suggest that CD2.6D79 with two more negatively charged residues enhances its binding to its target molecule CD48. This threefold increase in binding affinity is significant, since the majority of charged ligand mutants reported to date, except R87A (fivefold increase), show either a decrease or less pronounced affinity change (Davis et al. 1998; Kim et al. 2001).

Discussion

Common metal binding properties for naturally occurring Ca^{2+} -binding proteins

Ca^{2+} regulates many biological processes through interactions with proteins having different conformational, dynamic, and metal binding properties. Our laboratory has previously reported that different classes of Ca^{2+} -binding sites in naturally occurring Ca^{2+} -binding proteins can be identified and described using the popular pentagonal bipyramidal geometry in combination with the side chains of Asp, Glu, Asn, Gln, and main chain oxygen in the protein. In principle, residues located at the adjacent β -strands are capable of satisfying the geometrical

Table 2. NMR chemical shifts of selected residues of CD2.6D79

Residue	Distance (Å) ^a	Chemical shift		
		EGTA	Δ Chemical shift Ca^{2+}	Δ Chemical shift La^{3+}
R31	11.9	9.398	-0.045	-0.105
W32	11.3	9.459	0	0.172
E33	9.9	9.802	0	-0.006
E41	16.2	9.622	-0.178	-0.249
Y76	8.9	10.02	0.01	0.083
N77	6.6	9.618	0.001	0.039
D79	7.5	8.475	-0.027	-0.083
N90	6.7	8.340	-0.041	-0.11
K91	4.3	8.724	0.013	0.053
E92	4.2	8.396	-0.03	-0.099

^aDistance between the amide proton of a residue and the metal center in the model structure of CD2.6D79.

Figure 4. Mn^{2+} relaxation of the HSQC spectra. (A,B) HSQC spectra of the protein in the absence (black) and presence (red) of $10\ \mu\text{M}\ \text{Mn}^{2+}$. (C) Correlation of relaxation rate to amide distance of residues A9, F21, E5, D71, D79, V80, N90, E92, and K91 at $10\ \mu\text{M}\ \text{Mn}^{2+}$.

requirement for a Ca^{2+} -binding site (Fig. 1A). It was quite puzzling why nature does not use this form of pocket for Ca^{2+} -binding given the large range of affinities and the diversified roles of Ca^{2+} in regulating numerous biological functions. Even for the naturally occurring Ca^{2+} -binding proteins with a weak affinity (K_d at $0.1\text{--}3\ \text{mM}$), Ca^{2+} ions are largely chelated by the residues at the loop regions. For example, the Ca^{2+} -binding site in concanavalin A, which also has a net charge of -2 and a Ca^{2+} affinity similar to CD2.6D79, is formed by residues D19, D10, N14, and Y12 from the loop regions (Bouckaert et al. 2000).

Implications of the designed novel Ca^{2+} -binding protein

The design of CD2.6D79, whose Ca^{2+} -binding site is composed of ligand residues only from the β -sheets, reveals several important features for Ca^{2+} -binding. First, introducing the clustered charged ligand residues for Ca^{2+} -binding results in a large decrease in thermal stability although the overall structural, conformational, and folding properties of the host protein are largely retained (Figs. 1, 2). As revealed by electrostatic calculations, the charge repulsions between the ligands and the neighboring residues, such as E33, significantly reduced the electrostatic potential. Further, in the absence of Ca^{2+} , the fixed backbone conformation of the β -sheets does not allow for a decrease in the charge repulsion through conformational change. It also limits the use of local conformational change to complete neutralization of CD2.6D79 by Ca^{2+} -binding. Hence, Ca^{2+} -binding only leads to a small increase in the thermal stability. Consistently, a greater gain of the protein stability was achieved by binding of La^{3+} and Tb^{3+} (Table 1) with a higher positive charge.

Second, Ca^{2+} -binding to the β -sheet location has a less profound effect on the conformation of the protein than that of previously investigated Ca^{2+} -binding proteins (Chazin 1995; Ikura 1996; Ubach et al. 1998; Alattia

et al. 1999; Garcia et al. 2004; Yang et al. 2005b). Ca^{2+} -binding to the designed metal-binding site in CD2.6D79 was validated by Mn^{2+} relaxation methods (Fig. 4). However, the Ca^{2+} - or La^{3+} -induced chemical shift changes for the residues at and around the Ca^{2+} -binding pocket are very limited, $<0.05\ \text{ppm}$ and $0.10\ \text{ppm}$, respectively, and even smaller than that for residues at the flexible loop regions such as E41 (Fig. 3B), suggesting that their shielding environments were not perturbed by Ca^{2+} -binding. This is significantly different from the commonly observed Ca^{2+} -induced conformational changes for the intracellular (CaM and TnC) (Chazin 1995; Ikura 1996) or extracellular (cadherin and C2 domain) (Alattia et al. 1999; Garcia et al. 2004) proteins. Relatively large conformational changes in the Ca^{2+} -binding loop regions were still observed in the buffer proteins calbindin $_{\text{D9k}}$ and parvalbumin (Chazin 1995; Ikura 1996). NMR relaxation studies of the EF-hand motif have shown that the flexibility of Ca^{2+} signaling proteins is important for the modulation of Ca^{2+} -binding and function-specific motion (Capozzi et al. 2006). The structural movements have been expanded through evolution by connecting the duplicated domain with a flexible linker, which both increases the number of movements and the number of possible combinations between the protein and the target surfaces (Capozzi et al. 2006). Similarly, in our previously designed Ca^{2+} -binding protein, Ca.CD2, significant chemical shift changes ($>0.1\ \text{ppm}$) were observed for the two ligand residues in the loop regions (N60 and D62), while no significant change was detected for the ligand residue D15 at the β -strand B. This observed differential response to Ca^{2+} -binding is structurally specific since Ca.CD2 has a 2.5-fold weaker Ca^{2+} -binding affinity than that of CD2.6D79.

The large energetic cost in destabilizing the protein both in apo- and loaded-forms and the lack of a Ca^{2+} -induced conformational change explain why nature does

not generate Ca^{2+} -binding sites with only β -sheet ligand residues. The observed strong preference of Asp and Glu in the naturally occurring Ca^{2+} -binding proteins is consistent with the electrostatic nature of Ca^{2+} -binding, since almost all the binding sites observed to date have at least two negatively charged ligand residues. The use of the ligand residues predominantly from the loops and flexible regions of the proteins serves to minimize the repulsion between charged ligand residues in the apo-form and maximize the electrostatic interaction of the loaded-form by conformational change. Hence, our design studies and structural analysis provide a close view for the structural determinants that are necessary for biological functions of Ca^{2+} -binding in regulation and stability.

Implication for the electrostatic contribution on cell adhesion

The functional roles of CD2 in initiating a cellular immune response require a quick, reversible, and specific interaction with its target (CD48 for rat and CD58 for human CD2) (Blattman and Greenberg 2004). Hence, differing from the strong interactions often observed in enzyme-inhibitor and antibody binding (nM), CD2 binds to CD48 with a fast off rate that limits its affinity to the micromolar range. The large number of charged residues in the cell adhesion surface, in addition to shape complementary, were proposed to be essential for target specificity and less so for affinity (Davis et al. 1998). Most single mutations of charged residues on CD2 (to Ala or an opposite charge) reduce (or have no effect) on ligand binding except R87A (CD2.R87A), which results in a fivefold increase (Davis et al. 1998). Interestingly, a threefold decrease in the dissociation constant for CD48 binding was observed for CD2.6D79 compared with CD2 in the absence and presence of Ca^{2+} or La^{3+} (Fig. 2D). The results suggest that the CD48-binding ability of CD2 is enhanced by altering the electrostatic environment with the introduction of two charged mutations.

Figure 1, B and C, show the electrostatic potential of residue R87 on the ligand-binding surface of CD2, CD2.R87A, and CD2.6D79. The mutation in CD2.R87A reverses the potential of residue 87 from positive in CD2 to negative. A similar decrease in potential is observed at residue R87 in CD2.6D79 with the introduction of two negatively charged Ca^{2+} -binding residues. Calculations on the single mutants indicate that this change is due to the T79D mutation rather than the A92E mutation, which are 3.2 and 10.7 Å away from R87, respectively. Consistent with previous studies that indicate the charge at residue 87 is important for CD48 binding, changes in electrostatic interactions introduced by the charged mutations may be responsible for the increased binding affinity of CD2.6D79 to CD48. Long-range electrostatic

steering has been shown to be important in molecular recognition systems, including the interaction between the extracellular ribonuclease barnase and its natural inhibitor barstar. The binding sites of barnase and barstar have complementary charges, and the charge distributions are optimized for tight binding (Lee and Tidor 2001). Charged residues close to the binding site greatly accelerate barnase–barstar association.

Electrostatic switches have often been observed or postulated to regulate ligand binding, including phosphorylation/dephosphorylation, lipid and membrane binding, and protein–protein interactions. For example, the dynamic and weak binding to lipid membranes by C2 domain proteins, which also have an Ig-like fold similar to that of CD2, requires the neutralization of the negatively charged protein surface by Ca^{2+} -binding. The relatively low binding affinity and very fast off rate of CD2 binding to CD48 allows for reversible cell–cell interactions (McAlister et al. 1996). Dynamic and weak protein–protein interactions (K_d at micromolar instead of nanomolar range) usually involve interaction surfaces of 500–1000 Å² (Nooren and Thornton 2003). Interestingly, Ca^{2+} -binding does not have a significant effect on the CD2.6D79 binding to CD48. This is consistent with the fact that the designed site is outside of the CD48 binding region, and suggests that Ca^{2+} -binding does not disturb the electrostatic changes introduced in CD2.6D79 that are responsible for enhanced CD48 binding. Figure 1C shows that two charged mutations, A92E and T79D, have very different effects on the electrostatic potential at the cell adhesion “hot spot,” R87. While mutation A92E has virtually no effect on the charge environment at R87, mutation T79D has a large effect, suggesting that the charge effects at this region are very sensitive to the distance. It is possible that the distance between the bound Ca^{2+} and the cell adhesion surface is too far to result in significant effects on CD48 binding. Consistent with our experimental observation, the calculated potential of R87 in the Ca^{2+} -loaded form has a positive value (0.2 kcal/mol) like wild-type CD2 (1.38 kcal/mol). However, a large error may be associated with the estimation of the interaction energies using continuum methods due to the small position changes that occur with protein–ion interactions (Schymkowitz et al. 2005), and therefore the calculation on the Ca^{2+} -loaded form may have a large error. Further analysis of how Ca^{2+} -binding affects biological function would benefit from the development of force fields that accurately measure metals in continuum methods (Schymkowitz et al. 2005). Detailed examination of the binding characteristics of CD2 and CD48 will provide insight into the nature of cell–cell recognition, since it is typical of the molecular interactions mediating this process. Currently, we are investigating the contribution of the protein environment to Ca^{2+} -binding

and molecular recognition using the reported design approach.

In summary, we have successfully introduced a novel Ca^{2+} -binding site at an all β -sheet region on the molecular-recognition surface of CD2. The protein, CD2.6D79, binds to Ca^{2+} as well as its analogs Tb^{3+} and La^{3+} and maintains a wild-type-like structure. First, the small Ca^{2+} -induced conformational changes and the decreased thermal stability caused by the all β -sheet Ca^{2+} site reveal the structural requirement of Ca^{2+} -binding in regulating biological processes. The results of this study demonstrate why naturally occurring calcium-binding proteins frequently use flexible secondary structures to minimize destabilization caused by electrostatic repulsion of charged ligand residues. In addition, such information is useful for the future design of calcium-modulated proteins that can function as biosensors and calcium-switchable enzymes (Corey and Corey 1996; Pinto et al. 1997; Wang et al. 1999; Benson et al. 2001; Bolon and Mayo 2001; Di Costanzo et al. 2001; Lu et al. 2001). Further, the CD48-binding affinity of CD2 was increased with the introduction of the negatively charged residues, supporting a role for long-range electrostatic interactions in modulating cell adhesion. Such knowledge can be used for the further enhancement of cell adhesion with potential applications in immune therapy, tissue remodeling, and bone regeneration.

Materials and Methods

Rational design of a Ca^{2+} -binding site and protein engineering

A computer algorithm was used to design isolated Ca^{2+} -binding sites in a scaffold protein as previously described (Yang et al. 2003, 2005b). CD2.6D79 was selected based on the criteria described in the Results section.

Protein engineering, expression, and purification

Site-directed mutagenesis was used to engineer a single Ca^{2+} -binding site into CD2. CD2.6D79 was expressed in LB medium as a fusion construct with the enzyme Glutathione-S-Transferase (GST) in a pGEX-2T plasmid vector transformed into *Escherichia coli* BL21 (Yang et al. 2001). SV minimal media was used for the expression of ^{15}N -labeled protein. The protein was purified as a GST fusion protein using Glutathione Sepharose 4B beads (Pharmacia). The GST tag was cleaved using thrombin protease and further purified using a Superdex 75 column followed by a Hi trap SP column with a pH gradient. The protein concentration was calculated utilizing the molar extinction coefficient of $11,700 \text{ cm}^{-1} \text{ M}^{-1}$ for CD2 (Driscoll et al. 1991).

Far-UV CD

Excess metal was added to a 3- μM protein sample in 10 mM Tris, pH 7.4. Each protein sample was equilibrated for 20 min.

The far-UV CD spectra of the designed proteins were obtained from 190 to 260 nm with a bandwidth of 0.5 nm at room temperature using a Jasco 810 spectropolarimeter. A cell with light path of 1 cm was used. For thermal denaturation studies, two 20- μM protein samples were prepared in 10 mM Tris, pH 7.4 in the presence of excess Ca^{2+} (10 mM) or excess EGTA (1 mM). The samples were allowed to equilibrate for 20 min. The samples were monitored at 222 nm from 5°C to 100°C with a 10-min equilibration time at each temperature.

Tb^{3+} fluorescence

A 3- μM protein sample was prepared in 100 mM MOPS, pH 6.6. The metal titration was completed by increasing the metal:protein molar ratio directly to the protein sample. A 15-min equilibration time was allowed after each addition of metal. Competition assays were performed by titrating the desired metal to a pre-equilibrated Tb^{3+} and protein (3 μM) solution. After each addition, a 15-min equilibration time was allowed before the sample was measured. All Tyr/Trp-sensitized fluorescence resonance energy transfer experiments were performed using a PTI fluorimeter with 8 and 12 nm for the excitation and emission bandwidths, respectively. The emission spectra were collected from 500 to 600 nm with an excitation wavelength at 282 nm. The dissociation constant for Tb^{3+} was determined using the fractional change of the fluorescence intensity at 545 nm (Yang et al. 2003). All other dissociation constants were calculated using the Spec-fit 32 program (SPECFIT Global Analysis System, 2.10 [rev. X] ed.).

For selectivity experiments, the protein was incubated with 30 μM Tb^{3+} . The metal of interest was added to each protein-Tb sample to final concentrations of 100 μM La^{3+} , Mn^{2+} , Ni^{2+} , and Co^{2+} and 10 mM Ca^{2+} , Mg^{2+} , and K^{+} . Metal concentrations of 100 μM were used to minimize protein precipitation.

NMR spectroscopy and assignment

One-dimensional ^1H NMR spectra were recorded on a Varian Unity plus 500-MHz NMR spectrometer with a spectral width of 6600 Hz and complex data points of 17K using a modified WATERGATE pulse sequence at 25°C. A protein stock of 120 μM was prepared in 20 mM MOPS, 10 mM KCl, pH 6.8 with 5% D_2O . The data were processed with the program FELIX98 (MSI). Before Fourier transformation, a window function of 2 Hz exponential was used. All 2D NMR experiments were recorded on a Varian Unity plus 600-MHz NMR spectrometer with a spectral width of 8000 Hz. The samples were in 20 mM PIPES, 10 mM KCl, pH 6.8, with either EGTA (5 mM) or Ca^{2+} (10 mM). The protein concentrations were 1 mM for the homonuclear 2D TOCSY and NOESY experiments. TOCSY spectra were recorded with mixing times of 36, 60, and 90 s. NOESY spectra were recorded with mixing times of 100 and 150 s. The assignment of NMR spectra was achieved by standard methods and procedures (Yang et al. 2005b). For Mn^{2+} relaxation experiments, the protein was saturated with 10 mM Ca^{2+} , and increasing concentrations of Mn^{2+} (10, 20, 30, and 50 μM) were added.

Surface plasmon resonance

Surface plasmon resonance studies were performed at 25°C and a flow rate of 10 $\mu\text{L}/\text{min}$ using a Biacore 2000 (Biacore AB) in

HBS buffer (10 mM HEPES, 150 mM NaCl, pH 7.4) containing either 1 mM EGTA or 1 mM CaCl_2 . The anti-GST antibody was coupled to a CM5 sensor chip (Biacore) using the Amine Coupling Kit (Biacore) as directed, with the following modifications: After an activation step of 300 s, anti-GST antibody (30 $\mu\text{g/mL}$ in sodium acetate pH 5.0) was injected for 300 s. Immobilization levels were $\sim 17,000$ Response Units (RUs). The anti-GST antibody was regenerated with a 180 s injection of 10 mM glycine-HCl pH 2.0 (Biacore). The CD2-GST fusion proteins were diluted 1/10 in HBS buffer and injected for 180 s over immobilized anti-GST antibody. Soluble rat CD48 was diluted to 70 μM , and seven twofold dilutions thereof were injected for 30 s. Affinity constants were determined by non-linear fitting of the Langmuir binding isotherm to the data.

Electrostatic potential calculations

The electrostatic potentials were calculated using the program DelPhi (Nicholls and Honig 1991; Rocchia et al. 2001, 2002). The structure used in the calculation for CD2 was the PDB file 1hng (Chen et al. 1998), and for CD2.6D79 the model structure generated from the design process was used. The structures of CD2.R87A, CD2.T79D, and CD2.A92E were generated using SYBYL. The hydrogen atoms were built in and all structures were minimized with an Amber force field. For the DelPhi calculations, interior and exterior dielectric constants of 2 and 80, respectively, were used. The salt concentration was 0, and the linear solution of the Poisson-Boltzmann equation was imposed until convergence was reached.

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