Erratum

Structural and Functional Analysis of the Costimulatory Receptor Programmed Death-1

In this article (Immunity 20, 337–347, March 2004), the authors inadvertently omitted the names of two authors; there was also an omission from the Acknowledgments. The corrected sections follow.

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Structural and Functional Analysis of the Costimulatory Receptor Programmed Death-1

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Summary

PD-1, a member of the CD28/CTLA-4/ICOS costimulatory receptor family, delivers negative signals that have profound effects on T and B cell immunity. The 2.0 Å crystal structure of the extracellular domain of murine PD-1 reveals an Ig V-type topology with overall similarity to the CTLA-4 monomer; however, there are notable differences in regions relevant to function. Our structural and biophysical data show that PD-1 is monomeric both in solution as well as on cell surface, in contrast to CTLA-4 and other family members that are all disulfide-linked homodimers. Furthermore, our structure-based mutagenesis studies identify the ligand binding surface of PD-1, which displays significant differences compared to those present in the other members of the family.

T cell activation and regulation are critically dependent on signals provided by members of the CD28/CTLA-4 costimulatory receptor family. For example, CD28 and ICOS, upon engaging their respective ligands (B7-1/ B7-2 and B7RP-1), deliver stimulatory signals that upregulate the activity of the responding T cells (Carreno and Collins, 2002). In contrast, the binding of CTLA-4 to the B7-1/B7-2 ligands negatively regulates T cell activity, resulting in decreased T cell proliferation and decreased cytokine production. Programmed death-1 (PD-1) is another member in this family, sharing ~20% sequence identity with CD28, CTLA-4, and ICOS. Currently, the only two known sequences of PD-1 are the murine and human orthologs that share ~60% identity (Finger et al., 1997). The engagement of PD-1 by its specific ligands, B7-H1 (PD-L1) (Dong et al., 1999) or B7-DC (PD-L2) (Tseng et al., 2001), was reported to inhibit T and B cell proliferation and cytokine production (Freeman et al., 2000; Latchman et al., 2001; Okazaki et al., 2001). The inhibitory signals of PD-1 are mediated, at least in part, by the SHP-2 phosphatase, which is recruited to the phosphorylated Tyr residue in the immuno-tyrosine based switch motif of the cytoplasmic tail of PD-1 and dephosphorylates signal transducers of the TCR or BCR pathways (Latchman et al., 2001; Okazaki et al., 2001).

A critical role for PD-1 in immune regulation is highlighted by recent gene disruption studies that demonstrated strain-specific phenotypes: PD-1-deficient C57BL/6(B6) mice develop lupus-like autoimmune proliferative arthritis and glomerulonephritis with IgG3 deposition (Nishimura et al., 1999), while deficiency of PD-1 in BALB/c mice results in a severe autoimmunelated cardiomyopathy followed by death due to congestive heart failure (Nishimura et al., 2001).

PD-1 and the other receptors in this family are all type I transmembrane glycoproteins composed of an Ig Variable-type (V-type) extracellular domain, a transmembrane domain, and a cytoplasmic tail responsible for the binding of signaling and scaffolding molecules. CD28, CTLA-4, and ICOS are covalently linked homodimers due to the interchain disulfide mediated by the equivalent of Cys122 in CTLA-4 (Schwartz et al., 2002). In contrast, PD-1 cannot form such a covalent dimer as it lacks the analogous Cys residue and it is not known whether it forms noncovalent oligomers. In addition, PD-1 possesses only a single intrachain disulfide in its Ig V-type domain, while CD28, CTLA-4, and ICOS all contain two intrachain disulfides. Another distinct feature of PD-1 is that the CDR3 loop does not contain the conserved XFRPP(F/Y) motif present in CD28, CTLA-4, and ICOS, which is known to be critical for their ligand binding activities.

Consistent with this sequence disparity, PD-1 does not bind B7-1, B7-2, or B7RP-1, and it has not been established whether the CDR3 loop of PD-1 plays a role in binding its own ligands B7-H1 and B7-DC (Latchman et al., 2001; Sharpe and Freeman, 2002). Furthermore, in contrast to other receptors in this family that are only present on T cell surfaces, PD-1 is also expressed on the surface of activated B cells and plays a role in B cell regulation (Agata et al., 1996). Taken together, these distinct features of PD-1 set it apart as a unique member of the costimulatory receptor family. Currently, there are no data bearing on several important issues relevant to the structure and function of PD-1, including the structural similarities and differences between PD-1 and other costimulatory receptors, the ligand binding sites of PD-1, the oligomeric state of PD-1, and the signaling mechanism utilized by the complex of PD-1 and its ligands.
The extracellular Ig V-type domain of murine PD-1 was expressed in *E. coli* and refolded in vitro. This purified protein chromatographed as a symmetric monodisperse peak on a calibrated Superdex G-75 (30X1.0) gel filtration column. The elution time of 17.5–18 min corresponds to a molecular weight (MW) of ~15 kDa (Figure 1A), which is very close to the calculated MW of 13296 Da for the recombinant PD-1 monomer. The similarly refolded, purified human PD-1 protein (calculated MW = 14501 Da) also ran as a ~15 kDa monomer on Superdex G-75 gel filtration chromatography (data not shown).

PD-1 Is Monomeric when Expressed on the Cell Surface

To examine the oligomeric state of PD-1 in a cellular context, the coding regions for the cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP) were fused to the 3′ end of the full-length murine PD-1 gene. These constructs were cotransfected into CHO cells and the oligomeric state of the full-length protein on the cell surface was evaluated using FRET by photobleaching (Figure 2A). The tandem CFP-YFP fusion protein and full-length disulfide-linked dimeric murine CTLA-4 served as positive controls and gave a broad distribution of FRET efficiencies with averages of 24% ± 8% and 16% ± 5%, respectively (Figure 2B). The data scatter is typical of the CFP/YFP FRET pair (Pentcheva and Edidin, 2001). The FRET efficiency for the tandem CFP-YFP and disulfide-linked CTLA-4 showed no dependence on acceptor fluorescence intensity (Figure 2C). This behavior is characteristic of a system containing preassociated (i.e., clustered) donor/acceptor molecules, and is fully consistent with the covalent structure of these molecules (Kenworthy and Edidin, 1998). The distribution of FRET efficiencies for the full-length PD-1 constructs is greatly skewed toward lower values (relative to the positive controls) with an average value of 6% ± 4%, slightly but significantly higher than the FRET observed for the negative control (Figure 2B). FRET between CFP-PD-1 and YFP-PD-1 molecules showed a linear dependence on acceptor fluorescence intensity (Figures 2B and 2C). This is characteristic of FRET between randomly distributed molecules and is consistent with the presence of unclustered (i.e., monomeric) PD-1 on the cell surface (Kenworthy and Edidin, 1998). A short form of PD-1 with the cyto
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Figure 2. Cellular Behavior of PD-1

(A) Images of CFP and YFP fluorescence on individual cells pre- and postbleaching. For clearer presentation, the color scheme of the images is reversed with CFP and YFP displayed in red and blue, respectively.

(B) Distribution pattern of FRET efficiency (x-axis) as a function of number of cells (y-axis) for various groups as labeled. These data are pooled from four independent experiments, with total 14, 15, 21, and 25 cells examined for the tandem CFP-YFP, CTLA-4, L-PD-1, and S-PD-1, respectively. NC, negative control.

(C) The correlation between the acceptor intensity (expressed as arbitrary units) and the FRET efficiency for individual regions of interest (ROI) is shown for the groups as marked. The total number of ROI analyzed for each group is 60 to 80. The line corresponds to the best linear fit and its slope is the measure of dependence of FRET efficiency on the concentration of acceptor molecules (expressed as acceptor intensity) (Kenworthy and Edidin, 1998).

plasmic tail trimmed to 10 residues displayed spectral properties similar to the long form (Figures 2B and 2C), suggesting that the behavior of the full-length wild-type murine PD-1 is not due to the long (94 amino acids), and likely flexible, cytoplasmic tail. Together the data strongly support the idea that PD-1 is predominantly present as a monomer on the cell surface.

Binding Affinity of the Murine PD-1/B7-H1 Complex

In order to test whether the recombinant PD-1 produced in bacteria retains its biological activity, the interaction between murine PD-1 and the B7-H1-Ig fusion protein was measured by surface plasmon resonance (SPR) using a Biacore X instrument. The results demonstrate that our recombinant murine PD-1 specifically binds B7-H1-Ig coupled to CM5 sensor chips, suggesting that PD-1 refolded from inclusion bodies is biologically active. Equilibrium binding assays were performed to determine the equilibrium dissociation constant (Kd) between murine PD-1 and the B7-H1-Ig fusion protein. Murine PD-1, over a wide range of concentrations (78.0–0.5 μM), was sequentially injected over the sensor chip coupled with murine B7-H1-Ig (Figure 3A). At all concentrations examined, binding reached equilibrium within a few seconds, and similarly, complete dissociation was achieved within a few seconds after termination of the injections. These observations are consistent with fast koff and kon rates for the PD-1/B7-H1 interaction. Nonlin-
ear 1:1 Langmuir fitting of the binding data yielded a $K_d$ of $4.17 \pm 0.14 \mu M$, and linear fitting of the Scatchard plot of the same data resulted in a $K_d$ of $3.90 \pm 0.07 \mu M$ (Figure 3B). These equilibrium constants observed for PD-1/B7-H1 are comparable to the rest of the molecule, indicating this region of the binding data yield $K_d$ of 4.17.

The Structure of Murine PD-1 and a Comparison with that of CTLA-4

The PD-1 monomer displays a two-layer $\beta$ sandwich topology characteristic of Ig V-type domains, with the front and back sheets formed by the A’GFCC’ C’ and ABED strands, respectively (Figure 4A). Cys21 in the B strand and Cys90 in the F strand form an intrachain disulfide, which is a hallmark feature of Ig V-type domains (Metzler et al., 1997). An examination of contacts within the crystal does not identify any candidate dimer interface, consistent with the observed monomeric state of PD-1 in solution and on the cell surface.

Although there is only about 20% sequence identity between their extracellular Ig V-type domains, a superimposition of the structures of murine PD-1 and CTLA-4 monomers demonstrates that they have very similar overall structures as evidenced by the good overlay between most of the equivalent $\beta$ strands (Figures 5A and 5B) (Ostrov et al., 2000). This structural similarity is confirmed by the low root mean square (rms) deviation of ~1.5 Å between 76 equivalent $C_{\alpha}$ atoms from PD-1 and CTLA-4. However, there are significant differences between PD-1 and CTLA-4 involving the C' strand, C'C'D and CDR1 loops. The most pronounced difference is the placement of the C' strand that participates in the back face in CTLA-4 but participates in the front face in PD-1. Structural differences in the C'C'D and CDR1 loops between PD-1 and CTLA-4 are largely due to their different lengths as shown by the structure-based sequence alignment (Figure 5C).

Notably, nonpolar residues in the front sheet of PD-1 (M31, L32, A47, A48, I93, L95, P97, A99, and I101) form a large hydrophobic patch surrounded by hydrophilic residues, including charged residues D29, K45, K98, and E103 (Figures 4B and 4C), which is reminiscent of the organization present in classical protein-protein interfaces (Bogan and Thorn, 1998). In addition, these nonpolar residues are highly conserved between murine and human PD-1 (Figure 5C). These observations, in combination with the fact that other members in this family (CD28, CTLA-4, and ICOS) all use their front face for ligand binding (Schwartz et al., 2001; Stamper et al., 2001; Wang et al., 2002), suggest that this hydrophobic patch on the front face might be the predominant determinant for ligand binding in PD-1. Another hydrophobic patch on the surface of murine PD-1 formed by residues in the AA’ loop is involved in a crystal contact in the present crystal form. However, residues forming this hydrophobic patch are not conserved between murine and human PD-1 and there is no evidence supporting a role for this region in PD-1 function (Figure 5C).
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Figure 4. Structure of Murine PD-1

(A) Ribbon diagram of the Ig V-type domain of murine PD-1. The front (green) and back (aqua) sheets are composed of A′GFCCC″ and ABED strands, respectively. The CDR3 loop is labeled. The intrachain disulfide is shown in yellow. The four potential glycosylation sites are highlighted by showing the side chains of the Asn residues in red.

(B) Hydrophobicity of the molecular surface of murine PD-1. Hydrophobic residues are colored in yellow, whereas hydrophilic residues are colored in red. The orientation of the molecule is similar to that in (A). The large hydrophobic patch on the front face (circled) was hypothesized to be the ligand-binding site.

(C) Electrostatic molecular surface of murine PD-1. Negative and positive potentials are colored in red and blue, respectively. Charged residues surrounding the hydrophobic patch shown in (B) are labeled. The orientation of the molecule is similar to that in (A).

Increased hydrophobicity of the protein surface introduced by the mutation, since the Q5SS mutant refolded just as well as the wild-type. I93A had a significantly lower refolding yield compared to the wild-type and other mutants, but it was reasonably stable after refolding and adequately well behaved for binding assays. The lower refolding efficiency of I93A might be the consequence of a localized structural perturbation caused by the mutation, as I93 is located at the center of the hydrophobic patch. The purified HA-tagged wild-type and all the refolded mutants eluted as monodisperse peaks from the Superdex G-75 column at ~17 min, slightly earlier than the elution time of the nontagged PD-1 (not shown), consistent with the higher MWS of these epitope tagged proteins. Comparable refolding efficiency and similar behavior on gel filtration chromatography suggest that these mutants refolded correctly and have the same overall structure as the wild-type of murine PD-1. Therefore, the measured differences in binding affinities for the mutants, compared to the wild-type, should directly reflect the contributions of particular residues to the ligand binding activity of murine PD-1.

The binding activities of the HA-tagged and nontagged wild-type PD-1 were compared by injecting these two proteins at the same concentration over the same CM5 sensor chip coupled with murine B7-H1-Ig, which resulted in very similar response levels (data not shown). This result indicates that the HA-tag does not significantly affect the binding activity of PD-1 for its ligand. Therefore, all subsequent binding assays utilized the HA-tagged wild-type and mutants of murine PD-1. The wild-type and mutant proteins, at the same concentration, were sequentially injected over the sensor chips coupled with murine B7-H1-Ig fusion protein, and the response levels of the mutants were compared with that of the wild-type to obtain the relative ligand binding activities of the mutants.

The mutants can be divided into several groups based on their abilities to bind the B7-H1 ligand. K45A, I93A, L95A, I101A, and E103A have unmeasurable or extremely weak binding activity for the ligand compared to the wild-type (Figure 6; see Supplemental Table S2 at http://www.immunity.com/cgi/content/full/20/3/337/DC1), suggesting side chains of these residues are directly involved in and indispensable for the interaction between PD-1 and B7-H1. M31A, N33A, H96A, and K98A display significantly reduced binding compared to the wild-type (around 42%–67% of wild type), whereas N51A and L53A bind the ligand as well as the wild-type of PD-1 (Figure 6; Supplemental Table S2). Interestingly, D29A, D29S, and A99L display approximately 10%–20% higher binding to the ligand than the wild-type (Figure 6; Supplemental Table S2). The observation that the A99L mutation increased rather than abolished ligand binding was surprising, as it was predicted that the substitution of Ala with a bulky Leu residue would cause steric hindrance in the binding interface, and consequently, prevent PD-1 from interacting with B7-H1. However, considering the high inherent flexibility of the CDR3 loop suggested by the crystal structure, the bulky side chain...
Figure 5. Comparison of Murine PD-1 and CTLA-4

(A and B) Two orthogonal views of a superimposition of murine PD-1 (green) and CTLA-4 (gray) structures. Parts with significant differences between the two structures are labeled.

(C) Structure-based sequence alignment of PD-1 and CTLA-4. Identical residues between murine and human PD-1 are colored in yellow, whereas invariant residues between PD-1 and CTLA-4 are colored in red. Residues in murine PD-1 selected for mutation are highlighted with closed squares in blue. Residues in CTLA-4 involved in homodimerization and B7 binding (the MYPPPY loop) are highlighted with closed circles and closed squares, respectively. Cys122 mediating the interchain disulfide in CTLA-4 is highlighted with closed circles in green; a Glu residue occupies the equivalent position in PD-1.

of Leu99 might be readily accommodated by a local conformational adjustment upon the engagement of the ligand. Notably, the double mutant D29A/A99L exhibits approximately 35% higher ligand binding than the wild-type, suggesting an additive effect caused by the D29A and A99L single mutations (Figure 6E; Supplemental Table S2). Taken together, these results demonstrate that the residues altered in these mutants do not contribute significant binding energy to the PD-1/B7-H1 interaction. H74S and R81S, located on the back sheet and the interface between the front and back sheets, respectively, both exhibit ligand binding activities similar to the wild-type (Figures 6E and 6F; Supplemental Table S2). These observations are consistent with the conclusion that the ligand binding surface resides solely on the front face of PD-1 (Figure 7A).

Discussion

The present study shows that PD-1 is monomeric both in solution and on the cell surface, in contrast to CTLA-4, CD28, and ICOS, which are all obligate disulfide-linked homodimers (Carreno and Collins, 2002; Schwartz et al., 2002). The structures of the CTLA-4/B7 complexes (Schwartz et al., 2001; Stamper et al., 2001) and the disulfide-linked dimer of unliganded CTLA-4 (J.-C.D.S. and X.Z., unpublished data) have shown that the dimerization interface of CTLA-4 is formed by nearly invariant residues located C-terminal to the G strand and residues centered around the A’ strand. (Figure 5C). The analogous regions in PD-1 are not highly conserved between human and murine sequences (~60% identity in these regions versus ~65% identity in the whole extracellular domain) (Figure 5C), suggesting that these regions do not play a central role in the structure and function of PD-1. It should be noted that even in the absence of the interchain disulfide, a significant proportion of CTLA-4 exists as a noncovalent dimer in solution, demonstrating its inherent tendency to self-associate (Zhang et al., 2002). These observations imply an intrinsic difference between PD-1 and CTLA-4, and by extension, perhaps CD28 and ICOS as well. Importantly, the dimeric nature of the CTLA-4 and B7 molecules are critical features of the alternating lattice of the CTLA-4/B7 complexes that have been proposed to play a role in CTLA-4-mediated signal transduction (Schwartz et al., 2001; Stamper et al., 2001). Therefore, the monomeric nature of PD-1 suggests that this molecule might signal through a distinct
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Figure 6. Ligand-Binding Activities of the Murine PD-1 Mutants

The wild-type and mutants of HA-tagged murine PD-1 at 1.7 μM were injected at 20 μl/min for 2 min over CM5 sensor chips coupled with murine B7-H1-Ig fusion protein. The final response of each protein was calculated by subtracting the response of the control cell from that of the experimental cell. The response levels of the mutants were compared with that of the wild-type protein to obtain the relative ligand-binding activities of the mutants.

mechanism, such as ligand-induced dimerization, oligomerization, or conformational alterations. However, at present, a more detailed model of the signaling complex cannot be proposed since there are no data regarding the in vitro or in vivo oligomerization state of the PD-1 ligands. While the present studies demonstrate that a significant fraction of the PD-1 molecules exist as monomers on the cell surface, the spectroscopic techniques utilized cannot rule out the presence of a small and transient population of clustered PD-1 molecules, as has been recently demonstrated for CD40 (Reyes-Moreno et al., 2003).

Receptor/ligand binding assays demonstrate that bacterially expressed murine PD-1 lacking carbohydrate binds to murine PD-L1-Ig fusion protein with a Kd of ~4 μM. These observations demonstrate that, as has been previously shown for human CTLA-4 and B7-2 (Schwartz et al., 2001; Stamper et al., 2001; Zhang et al., 2002), glycosylation is not required for the ligand binding activity of PD-1. There are four potential glycosylation sites in the Ig V-type domain of murine PD-1. Three of these four sites, N16 (in the A’B loop), N41 (in the CC’ loop) and N83 (in the EF loop), are located distal to the ligand binding surface on the front face and most likely do not directly affect ligand binding of PD-1 (Figure 1A). However, glycosylation of these sites in vivo could provide restraints on the orientation of PD-1 on the cell surface and indirectly affect PD-1/PD-L interaction in the immunological synapse, as has been previously suggested for CD2 and ICAM-2 (Davis et al., 1998; Wang and Springer, 1998). The fourth site, N25, located in the CDR1 loop (BC loop) is in close proximity to the F and G strands, which are identified by our mutagenesis studies as major contributors to the ligand binding surface. Glycosylation of this site might influence the local structure, and consequently, could have some impact on the affinity of PD-1 for ligand. The qualitative features of the binding and dissociation phases of the sensorgrams suggest that the koff of the PD-1/B7-H1 interaction are rapid, similar to that observed for the CTLA-4/B7 complexes (Figure 3) (Collins et al., 2002).

The crystal structure of PD-1 provided critical information for rational mutagenesis studies, which resulted in an outline of the ligand binding surface on this costimulatory receptor. The hydrophobic residues identified to be critical for ligand binding (I93, L95, and I101) and charged residues (K45, K98, and E103) and two polar residues (N33 and H96) that are also involved in the PD-1/B7-H1 interaction (Figures 1 and 7). The hydrophobic center (I93, L95, and I101) and charged residues (K45 and E103) contribute significant binding energy to the receptor/ligand interaction, as indicated by the complete loss of ligand binding upon the mutation of these residues. Other polar residues surrounding the hydrophobic patch (N33, H96, K98) may mainly function in stabilizing the orientation between the receptor and ligand and determining the specificity of the interaction, as mutation of these residues results in reduced, but not abolished, ligand binding. The ligand binding surface in CTLA-4 is also composed of a cluster of hydrophobic residues (the MYPPPY CDR3 loop) and several hydrophilic residues (E33, R35, T53, and E97) (Figure 7B).
A more notable difference between the ligand binding forming a patch that is adjacent to the hydrophobic area.

A considerable flexibility with weak electron density and CCGTC3 of CTLA-4. In contrast, the CDR3 loop of PD-1 exhibits were 5 cis these residues are indispensable for ligand binding murine PD-1 cDNA as the template as described for ICOS by Wang ing interface and mutagenesis studies demonstrate that mutated to Ser was amplified by two-step PCR using the full-length CDR3 loop of CTLA-4 is a major contributor to the bind- The extracellular region of murine PD-1 with the unpaired Cys50

Figure 7. Comparison of Ligand-Binding Surfaces of PD-1 and CTLA-4

(A) Molecular surface of murine PD-1. Residues whose mutations resulted in abolished or significantly reduced ligand binding are colored in red, whereas residues whose mutations had no significant effects on ligand binding or led to higher ligand binding are colored in gray. The ribbon diagram is shown on the right to indicate the orientation of the molecule.

(B) Molecular surface of human CTLA-4 from the CTLA-4/B7-2 com-plex (Schwartz et al., 2001). Residues involved in ligand binding are colored in red. The ribbon diagram is shown on the right to indicate the orientation of the molecule. The CDR3 loops in both PD-1 (A) and CTLA-4 (B) are circled and labeled, showing the different contributions of this loop to ligand binding for these two receptors.

(Schwartz et al., 2001; Stamper et al., 2001). However, this surface of CTLA-4 is organized differently, in that the hydrophilic residues are located close to one another, forming a patch that is adjacent to the hydrophobic area. A more notable difference between the ligand binding surfaces of PD-1 and CTLA-4 is the role of the CDR3 loop in ligand binding. Structural studies reveal that the CDR3 loop of CTLA-4 is a major contributor to the binding interface and mutagenesis studies demonstrate that these residues are indispensable for ligand binding (Metzler et al., 1997; Morton et al., 1996; Schwartz et al., 2001; Stamper et al., 2001). The CDR3 loop of CTLA-4 adopts an unusual cis-trans-cis conformation and is very rigid, as shown by all the available crystal structures of CTLA-4. In contrast, the CDR3 loop of PD-1 exhibits considerable flexibility with weak electron density and relatively high B factors. Furthermore, mutagenesis studies show that residues in the CDR3 loop of murine PD-1 are not critical determinants for the PD-1/B7-H1 interaction. None of the four residues selected for mutagenesis (H96, P97, K98, and A99) in the CDR3 loop are absolutely required for ligand binding of PD-1 (Figure 7A; Supplemental Table S2). Consistent with their modest roles in ligand binding, residues in the CDR3 loop are not strictly conserved between murine and human PD-1 (Figure 5C). In contrast, the residues identified to be critical for ligand binding (K45, I93, L95, I101, and E103) are all contributed from strands in the front face.

Interestingly, a recent mutagenesis study on the ligands of murine PD-1 has also shown that residues from the strands on the front faces, but not those in the CDR3 loops, of both murine B7-H1 and B7-DC are required for binding PD-1 (Wang et al., 2003). Taken together, these mutagenesis data on both the receptor and ligands suggest that the complexes of PD-1 and its ligands adopt an orthogonal binding mode similar to that of the CD2/CD58 complex (Wang et al., 1999) such that strands from the front faces of the receptor and ligands make the major contribution to the receptor/ligand interface, whereas the CDR3 loops reside at the periphery of the interface and do not make critical atomic contacts. This interpretation suggests a strand-to-strand binding mode for the PD-1/PD-L complexes, which is distinct from the loop-to-strand binding mode observed in the CTLA-4/B7 complexes and also thought to be relevant to the CD28/B7 and ICOS/B7RP-1 costimulatory complexes (Schwartz et al., 2001; Stamper et al., 2001).

The mutagenesis study has also identified a few PD-1 mutants (D29A, D29S, A99L, and D29A/A99L) with slightly improved binding to the ligand compared to the wild-type. The observation of these moderately higher affinity mutants suggests the possibility of designing additional PD-1 mutants with even higher ligand binding affinities. Given the recent implication of PD-1 signaling in tumor immune evasion (Dong et al., 2002; Iwai et al., 2002), such reagents may prove valuable for the development of immuno-therapy against various malignancies.

In summary, PD-1 has been shown to be monomeric both in solution and when expressed on the cell surface. In addition, structure-based mutagenesis studies have identified the unique surface of PD-1 responsible for binding ligand. These findings suggest significant structural and functional differences between PD-1 and other members of the costimulatory receptor family.

Experimental Procedures

Cloning, Expression, and Purification of the Extracellular Ig V-Type Domains of Murine and Human PD-1

The extracellular region of murine PD-1 with the unpaired Cys50 mutated to Ser was amplified by two-step PCR using the full-length murine PD-1 cDNA as the template as described for ICOS by Wang et al. (2002). The flanking 5′ primer (incorporating an NdeI site and the start codon) and 3′ primers (incorporating a BamHI site) were 5′GGGCCTCAGATGTCCTCCATCCTCTCTCCACAGCCGG and 5′ACGGGATCTCTACTCCAGGATATTCTCCTGTTACAC, respectively. The two middle complementary primers containing the Cys to Ser mutation were 5′ACTGAAAACAGGCAGCCCTCCTCTAATGGTTTGAGCCAAC and 5′AACCGGGTGGTCTGACAAACATTAGAGAAGCGCC and 5′CTGTCTTTCAGT3′. The PCR product was sequenced and cloned
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into pET3a vector predigested with NdeI and BamHI as described for B7-1 previously (Zhang et al., 2002). Using this pET3a vector containing the PD-1 insert as the template, a HA-tagged derivative of wild-type murine PD-1 was amplified by PCR. The same flanking 5’ primer was used. The flanking 3’ primer (including the sequences that encode 3 linker residues and the HA tag) was 5’ GAGGTCA TATAG TGAACCCCCCACCCTCTCC3’ and 5’ GAGGTACCTCAAGGT GAGGGGCTGGGTGGCGC3’, respectively. The two middle complementary primers containing the Cys to Ser mutation were 5’ GCGAG CCACGCCGCAGCATGCTTGGTCGTAGGATCCTCAGGGT biosensor at 25°C. These experiments were performed using a Biacore X optical biosensor at 25°C. Research grade CMS sensor chips were activated by a 7 min injection of a 1:1 mixture of NHS and EDC from the amine coupling kit at a flow rate of 5 μl/min. 7–15 μl of mB7-H1-Ig fusion protein (50 μg/ml) in 10 mM acetate buffer (pH 5.0) were injected over the experimental flow cell and immobilized to the sensor surface by free amine coupling chemistry, which typically resulted in immobilization levels of 3500–4000 RU. mB7-H1-Ig (1 μM) was then injected for 7 min to deactivate both the experimental and reference flow cells. A sensor chip coupled with a misfolded murine B7-DC protein produced in the lab was used as a negative control for the binding assays. For determining the binding affinity of the wild-type murine PD-1 for mB7-H1-Ig, 16 serial dilutions of the PD-1 protein, starting from 78.0 μM to 0.15 μM, were injected into the negative control sensor chip, which resulted in response levels of 3500–4000 RU. These results confirm that the deactivation level of the chip is specific. Murine PD-1 was crystallized by the hanging drop vapor diffusion method by mixing 2 μl of crystal solution between various groups. The cross-species interaction of human PD-1 with murine B7-H1-Ig fusion protein was measured using the same protocol. The binding assays were performed on two flow cells at a flow rate of 20 μl/min for 2 min. Background responses of the reference flow cell were subtracted from the total responses of the experimental flow cell to give the final response levels. Each experiment was repeated three times and the average was taken as the final result. Purified human B7-2-Ig V-type domain at various concentrations in HBS-EP buffer was injected as negative control, which resulted in response levels of ~0 RU. Murine PD-1 was injected to the negative control sensor chip, which also generated response levels of ~0 RU. These results confirm that the detected binding is the specific interaction between PD-1 and the ligand. Equilibrium dissociation constants were obtained by fitting the data using nonlinear 1:1 Langmuir binding model and linear 1:1 binding Scatchard plot. The following equations were used for the nonlinear and linear fits:

\[ R = \frac{R_{\text{max}}}{C + K_d} \]  

\[ \frac{R}{C} = \frac{1}{K_d} + \frac{R_{\text{max}}}{K_d} \]  

Where \( R \) is the bound analyte in RU, \( R_{\text{max}} \) is the maximum response level, \( C \) is the concentration of free analyte in μM, and \( K_d \) is the equilibrium dissociation constant in μM.

The HA-tagged mutants of murine PD-1 were injected and the response levels were compared with that of the HA-tagged wild-type protein injected at the same concentration to obtain the relative ligand binding abilities of the mutants. A protein concentration of 1.7 μM (0.025 mg/ml) was used in these assays because this concentration of wild-type protein yielded high enough, but far from saturating, levels of SPR response shown by the experiments for affinity measurement. All of the experiments were repeated at least two times and the differences between the repeats were negligible (<1%).

Crystallization, Data Collection, and Structure Determination

Murine PD-1 crystal was visualized by the hanging drop vapor diffusion method by mixing 2 μl of protein at 6 mg/ml and 2 μl of crystallization buffer composed of 20%–30% PEG 8000, 100 mM NaAc, and 100 mM NaCacodylate (pH 6.5). Crystals were flash cooled to 100 K under a stream of nitrogen gas in the crystallization buffer supplemented with 15% Glycerol before data collection. Diffraction data

FRIT Analyses for Murine PD-1 on the Cell Surface

Full-length murine PD-1 was amplified by PCR using a 5’-primer: 5’ ATGATTGCGCAGCATGCTTGGTCGTAGGATCCTCAGGGT biosensor at 25°C. These experiments were performed using a Biacore X optical biosensor at 25°C. Research grade CMS sensor chips were activated by a 7 min injection of a 1:1 mixture of NHS and EDC from the amine coupling kit at a flow rate of 5 μl/min. 7–15 μl of mB7-H1-Ig fusion protein (50 μg/ml) in 10 mM acetate buffer (pH 5.0) were injected over the experimental flow cell and immobilized to the sensor surface by free amine coupling chemistry, which typically resulted in immobilization levels of 3500–4000 RU. mB7-H1-Ig (1 μM) was then injected for 7 min to deactivate both the experimental and reference flow cells. A sensor chip coupled with a misfolded murine B7-DC protein produced in the lab was used as a negative control for the binding assays. For determining the binding affinity of the wild-type murine PD-1 for mB7-H1-Ig, 16 serial dilutions of the PD-1 protein, starting from 78.0 μM to 0.15 μM, were injected into the negative control sensor chip, which resulted in response levels of 3500–4000 RU. These results confirm that the deactivation level of the chip is specific. Murine PD-1 was crystallized by the hanging drop vapor diffusion method by mixing 2 μl of protein at 6 mg/ml and 2 μl of crystallization buffer composed of 20%–30% PEG 8000, 100 mM NaAc, and 100 mM NaCacodylate (pH 6.5). Crystals were flash cooled to 100 K under a stream of nitrogen gas in the crystallization buffer supplemented with 15% Glycerol before data collection. Diffraction data

**Analytical Ultracentrifugation Analyses for Murine PD-1**

Purified murine PD-1 was exchanged into a buffer containing 10 mM Tris-HCl and 100 mM NaCl (pH 8.0) using an Amicon concentra-
from a single crystal were collected to 2.0 Å resolution at beamline X8B (National Synchrotron Light Source, Brookhaven National Laboratory) using a 2X2 ADSC CCD Detector. The data were consistent with the orthorhombic space group P212121 (a = 23.97 Å, b = 50.80 Å, c = 75.28 Å). Data were processed with the HKL suite (Otwonowski and Minor, 1997) and data collection statistics are summarized in Supplemental Table S1.

A BLAST search of the NCBI database suggested that the Ig V-type domain of a TCRβ chain (Vβ-2.6) was the most homologous molecule to PD-1 (25% identity). The crystal structure of this Ig V-type domain (PDB Number 1888), with all of the nonidentical residues between the model and PD-1 mutated to Ala or kept unchanged if the original residue is a Gly, was used as the search model for molecular replacement trials performed with the program EPMR at 4.0 Å resolution (Kissinger et al., 1999). The best solution with a single monomer of the model molecule from molecular replacement has a correlation coefficient of 0.25 as calculated by EPMR. Rigid body refinement of this model at 2.2 Å resolution using the program CNS (Brünger et al., 1998) resulted in an Rfactor of 54.4%. Despite the high Rfactor, a 2Fobs-Fcal electron density map calculated at 2.2 Å resolution was readily interpretable, with continuous densities for most of the α strands. Modeling and refinement at 2.0 Å resolution (including torsion angle dynamic simulated annealing, bulk solvent correction, and individual B factor refinement) were performed by using the program O (Jones et al., 1991) and CNS, respectively. The final model contains all of the residues of murine PD-1, except the C-terminal residue Glu and the side-chains of His86, Pro87, and Lys100, and 73 H2O molecules, with Rcryst and Rfree of 19.82% and 22.83%, respectively. Structure refinement statistics are summarized in Supplemental Table S1.

Molecular superimpositions and rms deviation calculations were performed with FIT (G. Lu, http://bioinf1.mbfys.iu.edu/sequoung/fit.html). Structure-based sequence alignment was rendered with the program ESPript (Gouet et al., 1999). Molecular surfaces were generated with grasp (Nicholls et al., 1991) or Pymol (DeLano W.L., http://www.pymol.org). Ribbon diagrams were generated with Setor (Evans, 1993).

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References


Accession Numbers

Coordinates and structure factors have been deposited in the PDB under accession code 1NPU.