Conformational Rearrangement within the Soluble Domains of the CD4 Receptor Is Ligand-specific*

Ashish 1, Ignacio J. Juncadella 3, Renu Garg 1, Christopher D. Boone 1, Juan Anguita 1,1 and Joanna K. Krueger 1,2

From the Departments of 1Chemistry and 4Biology, University of North Carolina at Charlotte, Charlotte, North Carolina 28223 and the 3Department of Veterinary and Animal Sciences, University of Massachusetts at Amherst, Amherst, Massachusetts 01003

Ligand binding induces shape changes within the four modular ectodomains (D1–D4) of the CD4 receptor, an important receptor in immune signaling. Small angle x-ray scattering (SAXS) on both a two-domain and a four-domain construct of the soluble CD4 (sCD4) is consistent with known crystal structures demonstrating a bilobal and a semi-extended tetralobal Z conformation in solution, respectively. Detection of conformational changes within sCD4 as a result of ligand binding was followed by SAXS on sCD4 bound to two different glycoprotein ligands: the tick saliva immunosuppressor Salp15 and the HIV-1 envelope protein gp120. Ab initio modeling of these data showed that both Salp15 and gp120 bind to the D1 domain of sCD4 and yet induce drastically different structural rearrangements. Upon binding, Salp15 primarily distorts the characteristic lobal architecture of the sCD4 without significantly altering the semi-extended shape of the sCD4 receptor. In sharp contrast, the interaction of gp120 with sCD4 induces a shape change within sCD4 that can be described as a Z-to-U bi-fold closure of the four domains across its flexible D2–D3 linker. Placement of known crystal structures within the boundaries of the SAXS-derived models suggests that the ligand-induced shape changes could be a result of conformational changes within this D2–D3 linker. Functionally, the observed shape changes in CD4 receptor causes dissociation of lymphocyte kinase from the cytoplasmic domain of Salp15-bound CD4 and facilitates an interaction between the exposed V3 loops of CD4-bound gp120 molecule to the extracellular loops of its co-receptor, a step essential for HIV-1 viral entry.

The four extracellular soluble domains of CD4 (sCD4) interact with several physiological ligands, resulting in varied signals within the T cell (1, 2). Two such ligands, which are the focus of this study, are the tick saliva protein Salp15 and the HIV-1 viral entry protein gp120. Salp15 binding to CD4 impairs the ability of CD4+ T cells to produce interleukin-2 and become activated (3, 4). The activity of Salp15 may contribute to the efficient feeding of Ixodes scapularis ticks, which are the vectors of significant infectious diseases. Similarly, binding of the HIV-1 envelope glycoprotein gp120 to the D1 domain of CD4 is the initiating step of HIV-1 viral entry process leading to HIV-1 AIDS (5). Structural insight on the conformation of this receptor with and without such ligands may contribute significantly to our understanding of the biological events during the initiating steps of HIV-1 AIDS and tick-borne diseases.

The three-dimensional structures of unliganded sCD4s on a human D1–D2 (sCD4D1–D2) (6–9), a rat D3–D4 (sCD4D3–D4) (10), and a human D1–D4 (sCD4D1–D4) (2) construct demonstrate the relative orientations of the four Ig-like soluble domains and indicate that a flexible linker is present between the D2 and D3 subdomains. Furthermore, several crystal structures of sCD4D1–D2 in complex with the HIV-1 gp120 “core” (including neutralizing antibodies) have been published (11–14). The available crystal structures of human gp120 core-antibody/sCD4D1–D2 complexes, unliganded SIV core were used to provide interpretation of SAXS data-based models of sCD4D1–D2 ± full-length gp120, providing clear structural evidence that a major shape reorientation occurs in the gp120 portion of this sCD4D1–D2 complex (15). The crystal structure of a sCD4D1–D2 bound to a Class II major histocompatibility complex molecule also concluded that there are no large scale rearrangements within the receptor (16). All of these structures predicted small changes in the bilobal shape of the sCD4D1–D2 upon ligand binding.

Our current work adds to the structural understanding of HIV viral entry by providing evidence that upon interaction with the full-length fully glycosylated gp120, significant structural changes occur within the four sCD4 domains, likely via changes within the proposed flexible linker between domains 2 and 3. Further, these large scale changes within sCD4 were not observed upon binding of another of its ligands, the tick saliva protein Salp15. These results provide insight into how different downstream processes are affected by these two proteins, which bind the same region of the sCD4 receptor but elicit variable conformational responses within that receptor.

**EXPERIMENTAL PROCEDURES**

sCD4D1, D2 and sCD4D1, D4—The two constructs of the sCD4 protein, sCD4D1, D2 and sCD4D1, D4 were obtained through the National Institutes of Health AIDS Research and Reference Reagent Program. The D1–D2 construct includes the first 183 residues of human sCD4 and was produced in Escherichia coli.
SAXS-based Models of sCD4 ± (Salp15 or gp120)

Resultant inclusion bodies were refolded and purified as published (17). The recombinant human sCD4D1–D4 contains the first 370 amino acids of CD4, was expressed in COS cells, and contains post-translational glycosylation. Both forms of sCD4 were provided at a concentration of ~1 mg/ml and >95% purity.

HIV-1 gp120—The full-length, fully glycosylated gp120 was also obtained through the National Institutes of Health AIDS Research and Reference Reagent Program at a concentration of ~1 mg/ml and >95% purity. The recombinant protein was prepared from Chinese hamster ovary cells infected with macrophage tropic virus HIV-1 SF162.

Salp15—Salp15-V5/His was prepared from cultured Drosophila S2 cells as described previously (3, 4). Briefly, Drosophila S2 cells were grown as spinner cultures, switched to DES serum-free medium for 2 days, and induced with copper sulfate at a final concentration of 500 μM imidazole, dialyzed extensively against phosphate-buffered saline and reported by the National Institutes of Health AIDS Reference and Reagent Program. Actual concentrations of the unliganded proteins were later determined by comparing the observed I₀ values from their samples to that of a standard protein, lysozyme (see Table 1). This analysis indicated that the molar ratios of the complexed proteins were closer to 1.7 for Salp15:sCD4D1–D4:1.2 for Salp15:scD4D1–D4:1.3 for gp120:scD4D1–D4: and 1.1.8 for gp120:scD4D1–D4.

All of the mixtures were prepared 5–10 min prior to SAXS data collection. All of the experiments were carried out in duplicate.

SAXS Data Analysis—For a monodisperse sample of globular protein or its complex, a plot of ln(l(q)) versus q² where q = Rg / l₀ should be linear and will fit to the following equation.

\[ I(q) = I_0 e^{-l(2q)^{2}} / l_0 \]

(Salp15 or gp120)

Such analysis, known as Guinier analysis, provides an estimation of the scattering particle radius of gyration (Rg) and for- ward or zero-angle scatter (I₀) from the slope (Rg² / l₀) and the y intercept I₀ (19). Guinier analyses of all scattering data were done using the Primus software package (20). Indirect Fourier transformation of the scattering data over the measured q range gives a pair-wise distribution function of interatomic vectors, P(r). The inverse Fourier transform of I(q) yields P(r), which is the frequency of vector lengths connecting small volume elements within the entire volume of the scattering particle.
calculation was repeated five times, with no predefined shape or symmetry bias. The final models were obtained by averaging these five independent solutions using the DAMAVER suite of programs (23). Such averaged models provide uniform density models representing the “most probable” shape of the molecules in solution. To compare the SANS-based models of sCD4 (D1–D2 and D1–D4) with their previously known structures from crystallography data, the structures were overlaid using the SUPCOMB13 program (24) in an automated manner.

**Graphical Analysis and Representations**—The programs, RASWIN 2.6ucb, SPDB viewer, and WebLab ViewerPro 3.7 were used for graphical analysis, manual alignment, and figures generation.

**T Cell Activation**—Naïve splenic CD4⁺ T cells from C3H/HeN and Balb/c mice were purified by negative selection as described (3). Purified CD4⁺ T cells were preincubated with or without Salp15 (50 μg/ml) and stimulated for the indicated times at 37 °C with 10 μg/ml of anti-CD3ε, 1 μg/ml of anti-CD28, and 10 μg/ml of anti-armenian and Syrian hamster IgG (BD Biosciences, San Jose, CA).

**Western Blotting and Immunoprecipitation**—5 × 10⁶ purified CD4⁺ T cells were stimulated as before. The cells were lysed in 60 mM Tris, pH 7.4, containing 150 mM NaCl, 5 mM EGTA, 1 mM β-glycerol phosphate, 2.5 mM sodium pyrophosphate, 25 mM NaF, 2 mM sodium orthovanadate, 1% Triton X-100, 0.25 sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors for 15 min on ice; sonicated four times for 5 s; and incubated at 37 °C for 10 min.

### Table 1

**Summary of parameters calculated from the P(0) analysis of the SANS data acquired for unliganded receptor sCD4, Salp15, gp120 and their complexes**

| Sample | Mass (kDa) | Rₚ (Å) | Dmax (Å) | Ion (mg/ml) | Expected Ion⁻¹ | Concentration⁻¹ |
|--------|------------|--------|----------|------------|--------------|----------------|
| Standard (lysozyme) | 14.2 | 14.6 ± 0.1 | 44 | 9.7² | 17.8 | 1.25 |
| Unliganded receptor sCD4₁–D₂ | 26 | 22.3 ± 0.1 | 141 | 39.0 ± 0.5 | 30.7 | 1.26 |
| sCD4₁–D₄ | 45 | 38.1 ± 0.1 | 141 | 39.0 ± 0.5 | 30.7 | 1.26 |
| Salp15 ± sCD4 | 25 | 27.0 ± 0.7 | 101 | 9.2 ± 1.2 | 17.1 | 0.35 |
| Salp15 | 51 | 31.3 ± 0.1 | 104 | 12.1 ± 0.4 | 13 | 0.3 |
| Salp15/sCD4₁–D₂ | 70 | 43.8 ± 0.2 | 144 | 14.8 ± 0.6 | 18 | 0.29 |
| gp120 ± sCD4 | 120 | 43.7 ± 0.1 | 166 | 77.2 ± 0.2 | 82 | 0.95 |
| gp120/sCD4₁–D₂ | 146 | 44.4 ± 0.1 | 165 | 88.2 ± 0.2 | 95 | 0.69 |
| gp120/sCD4₁–D₄ | 165 | 49.3 ± 0.1 | 181 | 115.2 ± 0.2 | 113 | 0.93 |

² Expected Ion⁻¹ values are based upon the Ion measured for the lysozyme standard given the corresponding molecular weight and/or known concentrations and assuming 100% binding of the complexes.

³ Concentrations of the uncomplexed proteins were determined based upon their molecular weights and the measured Ion of the lysozyme standard. Concentrations for the complexes are actual concentrations calculated from the amounts of each protein used to make the complex and the concentrations determined for the individual proteins using Ion analysis.

⁴ The intensity at zero angles for 1 mg/ml of lysozyme was estimated by extrapolating Ion⁻¹ values obtained from four different lysozyme samples with predetermined concentrations.

⁵ These parameters have been reported earlier (15).
The cell lysate was cleared by centrifugation and boiled in SDS sample buffer. The samples were then subjected to SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with the indicated antibodies. For immunoprecipitation experiments, the cells were lysed in 300 μl of lysis buffer as above and incubated overnight at 4 °C with the indicated antibodies. 50 μl of 50% protein-A agarose slurry beads (Invitrogen) were added to the samples and incubated for 2 h at 4 °C. The beads were washed three times with cold lysis buffer, and SDS sample buffer was added and boiled. The immunoprecipitate was run in a SDS-PAGE following immunoblotting.

RESULTS AND DISCUSSION

Z-shaped sCD4D1–D4 with a Flexible D2–D3 Junction—The measured SAXS data from sCD4D1–D4 sample with a concentration of ~1 mg/ml is shown in Fig. 2A. The linearity of the Guinier plot, over a q range 0.01–0.025 Å⁻¹, reflects the monodisperse and globular nature of molecules (Fig. 2A, inset). The slope of the linear fit of Guinier analysis indicated an R_g of 37.9 ± 0.1 Å for the full-length sCD4 molecules in solution. In good agreement, the P(r) calculated considering SAXS data from 0.01–0.25 Å⁻¹, indicated that unliganded sCD4 molecules are particles with a D_max of ~140 Å and an R_g of 38.1 ± 0.1 Å (Fig. 2B). The analysis of the SAXS data acquired on a sCD4D1–D2 sample with similar concentration provided the R_g and D_max for this protein to be 22.3 and 75 Å, respectively (15). The P(r) calculated for the two-domain sCD4 construct is shown alongside the curve obtained for full-length receptor (Fig. 2B). It can be seen that the full-length sCD4 molecules containing all four domains are almost double in their maximum linear dimension than the two domain sCD4. Comparison of the observed Iₒ value of 39.0 ± 0.5 with that extrapolated for 1 mg/ml of standard protein hen egg white lysozyme confirmed that at a concentration of 1.2 mg/ml, sCD4D1–D4 molecules exist as monomers in solution (Table 1).

The crystal structure of unliganded human sCD4D1–D4 shows that the molecule adopts a semi-extended Z shape with dimeric contacts across its D4 domain (2). Using the glycosylated form of sCD4D1–D4, these structures have been solved in three different lattices (Protein Data Bank codes 1WIO, Tetragonal (P4 322); 1WIP, Monoclinic (P2(P121); and 1WIQ, Trigonal (P3121)). Comparison of inde-
ependent structures from these crystals (resolution range from 3.9 to 5 Å) identified a hingelike variability at the junction between the D1–D2 and the D3–D4 halves. This junctional flexibility, located at the Leu$^{177}$ and Ala$^{178}$ residues in the D2–D3 linker, was suggested to play an important role in immune recognition and HIV fusion (2), especially because both amino acids plus Val$^{176}$ and Leu$^{177}$ are conserved among several species including human, mouse, rat, rabbit, and cat (25).

The dimensions of the sCD4$^{D1-D4}$ molecules deduced from SAXS data analysis, at a concentration of ~1 mg/ml, were similar to those reported for a monomer of this glycoprotein from ultracentrifugation studies and from diffraction studies at modest resolutions (2, 26). The CRYSOL program computed values in the range of 1.1–1.5 when comparing the calculated I(q) from the individual monomeric chains from the three crystal structures of sCD4$^{D1-D4}$ with the measured SAXS data (27). This good correlation reflected the similarity between the predominant shape information present in the SAXS intensity data and the monomeric portion of the crystal data. Thus, as expected, structure restoration from SAXS data solved a semi-extended structure for the sCD4$^{D1-D4}$ molecules in solution (Fig. 3, A and B). The extra volume near the middle of the SAXS-based model that is unaccounted for when overlaid with the crystal structure of sCD4$^{D1-D4}$ could be reflective of the inherent structural variability in this region.

There are six structures of human sCD4$^{D1-D2}$ deposited in the Protein Data Bank (codes 1CDH, 1CDI, 1CDJ, 1CDU, 1CDY, and 3CD4) (6–9). The resolution of these structures ranges from 2 to 2.9 Å. Using CRYSOL (27), a comparison of the calculated SAXS I(q) profiles for each of these structures with our experimentally measured data resulted in a χ-value between 1.0 and 1.1, indicating that the shape information encoded in our SAXS data is consistent with the crystal structures. Fig. 3C presents the most probable solution structure of the sCD4$^{D1-D2}$ molecule obtained by averaging five models restored from SAXS data using the DAMMINIQ program (22). This model is similar to the chain ensemble model published earlier (15). Automated overlay of our model on the crystal structure of recombinant sCD4$^{D1-D2}$ (Protein Data Bank code 3CD4) confirms the expected good correlation between shape information in SAXS data and crystal data.
Importantly, the SAXS data analysis and structure reconstruction of sCD4 molecules conclude that 1) in absence of any ligand, the truncated sCD4D1–D2 receptor adopts an extended bilobal shape in solution and 2) despite the flexible nature of the D2–D3 junction, the relative positioning of D1–D2 and D3–D4 halves in the unliganded sCD4D1–D4 molecules in solution is nearly identical to that seen in its crystal structure.

Unliganded Tick Saliva Salp15—The Salp15-V5/His monomer was purified using a gel filtration column prior to its use in SAXS experiments. The low angle scattering intensity as a function of \( q \) is shown in Fig. 4A. The slope of the linear fit to the Guinier plot over the \( q \) range 0.011–0.044 Å\(^{-1}\) indicated an \( R_g \) of 26.7 ± 0.3 Å for this globular molecule (Fig. 4A, inset). The \( P(r) \) obtained from the indirect Fourier transformation of the SAXS data, over a \( q \) range of 0.011–0.25 Å\(^{-1}\), indicated that the predominant shape of this molecule in solution has a \( D_{max} \) and an \( R_g \) of 101 and 27 ± 0.7 Å, respectively (Fig. 4B and Table 1). The peak and shoulder observed in the \( P(r) \) profile for Salp15-V5/His molecules implied that the shape of this molecule is bilobal with the two lobes being asymmetric in size. Given a molecular mass of 25 kDa for the Salp15-V5/His glycoprotein, the \( I_0 \) values normalized to the value of a standard protein suggested that the sample of the Salp15 monomer had a concentration of 0.35 mg/ml (Table 1).

Ab initio structure restoration of the measured SAXS data provided dummy residue models of the Salp15 monomer (Fig. 5A). An average of five independent solutions resulted in a three-dimensional model of the predominant structure adopted by Salp15 molecules in solution (Fig. 5, B and C). As expected from the \( P(r) \) curve, the model is characterized by a maximum linear dimension of 101 Å and has two asymmetric globular domains, with ~75 and 25 Å in their long axis. Considering the functional inertness of the 23 amino acid residues belonging to the V5/His tags at the C terminus of Salp15, we assume that they reside in the volume of the smaller lobe and the linker connecting it to the main mass of the parent glyco-

**FIGURE 6. Most probable shape of the 1:1 complexes of Salp15-sCD4.** These plots show the comparison between the calculated \( I(q) \) profiles (line) of models restored for Salp15-sCD4\(_{D1-D2}\) (A) and Salp15-sCD4\(_{D1-D4}\) (B) restored from their measured SAXS data (gray circles). Two rotated orientations of the models representing the most probable shape of the 1:1 complex of Salp15-sCD4\(_{D1-D2}\) (C) and Salp15-sCD4\(_{D1-D4}\) (D) are presented in space-filled mode.
SAXS-based Models of sCD4 ± (Salp15 or gp120)

shape of the scattering particles have an $R_g$ of 31.2 ± 0.3 Å. The $P(r)$ solved by indirect Fourier transformation of the scattering data ($q$ range, 0.026–0.25 Å$^{-1}$) indicated that the predominant particle size in the Salp15-sCD4$_{D1-D4}$ mixture had a $D_{max}$ and $R_g$ of 104 and 31.3 ± 0.1 Å, respectively (Fig. 4D and Table 1). The observed $I_q$ value of 12.1 ± 0.4 indicated at least 90% 1:1 binding between the Salp15 and sCD4$_{D1-D2}$ molecules (Table 1).

Similarly, the SAXS data collected from a sample containing mixture of the Salp15 and sCD4$_{D1-D4}$ molecules is shown in Fig. 4A. Guinier analysis of the data over the $q$ range 0.011–0.029 Å$^{-1}$ indicated that the predominant scattering particle in this mixture has an $R_g$ of 43.1 ± 0.3 Å (Fig. 4A, inset). The $P(r)$ analysis of the SAXS data ($q$ range, 0.011–0.25 Å$^{-1}$) suggested that the predominant contributions to scattering arose from molecules with a $D_{max}$ and $R_g$ value of 144 and 43.8 ± 0.2 Å, respectively (Fig. 2D and Table 1). In comparison with the expected $I_q$ value of 18 for this mixture, we observed an $I_q$ value of 14.8 ± 0.6, which implies at least 85% binding between available Salp15 and sCD4$_{D1-D4}$ molecules (Table 1).

*Ab initio* structure reconstruction was carried out with the SAXS data obtained from the mixtures of Salp15 with both sCD4 (D1–D2 and D1–D4). Three-dimensional uniform density models were generated from the structure constraints encoded in the SAXS intensity profiles and reflected the predominant conformation from the ensemble of molecules in solution (Fig. 6, A and B). Because the intensity of scattering at low angles is proportional to the square of the molecular mass, the 1:1 complexes Salp15-sCD4$_{D1-D2}$ and Salp15-sCD4$_{D1-D4}$ will contribute ~80 and ~70% to the total scattering profile of their respective mixtures. In other words, the model solved from the SAXS data sets of the mixtures reflects the predominant solution conformation of the Salp15-sCD4 1:1 complexes. The black lines shown with the SAXS intensity data of the mixtures in Fig. 6 (A and B) are the calculated $I(q)$ profile for the reconstructed models. The average of the five independent models solved for the Salp15-sCD4$_{D1-D2}$ and Salp15-sCD4$_{D1-D4}$ complexes are shown in Fig. 6 (C and D), respectively. It is not straightforward to delineate which portion of the model resides the bound Salp15 or the domains of protein. Considering that the secondary structural content of this tag is composed of 100 and 50% mixture of $\alpha$-helix and $\beta$-sheet, the models had shapes with a $D_{max}$ of ~18–37 Å, which is comparable in dimensions with the smaller lobe seen in the SAXS-based models of Salp15.

1:1 Complex between Tick Saliva Salp15-sCD4 (D1–D2 and D1–D4)—Based on gel filtration results, we have earlier reported that monomeric Salp15 forms 1:1 complexes with both sCD4$_{D1-D2}$ and sCD4$_{D1-D4}$ in solution (4). The SAXS data acquired on the mixture of Salp15 and sCD4$_{D1-D2}$ is presented in Fig. 4A. For our analysis, we did not use the data below 0.026 Å$^{-1}$. The slope of the linear fit to the Guinier plot over the $q$ range from 0.026–0.041 Å$^{-1}$ suggested that the predominant

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**FIGURE 7.** Salp15 induces shape changes in sCD4, which eventually disrupts the interaction between CD4 and the Src kinase p56lck. A, two views show a manual overlay of the models computed for unliganded sCD4$_{D1-D2}$ and its complex with Salp15. B, two rotated views of the superimposition generated for the models solved for unliganded sCD4$_{D1-D4}$ and its complex with Salp15 are presented. C, the overlay generated by aligning the shape profile of the bound Salp15 in the models of the two complexes. D, murine CD4$^+$T cells were activated with 10 μg/ml of αCD3 and 1 μg/ml of αCD28 in the absence or presence of 50 μg/ml of Salp15 for 20 min. CD4 was immunoprecipitated (IP) from whole cell extracts and the levels of p56lck and CD4 (control) were analyzed by Western blotting (IB).
the sCD4 receptor. On comparing the structures of the two complexes in three dimensions, we found a lobe with \( \sim 57 \) Å in \( D_{\text{max}} \) on one end of both complexes.

Assuming that no additional structural rearrangement occurs when Salp15 binds with sCD4 containing D3 and D4 domains, it is likely that bound Salp15 might reside in this structurally similar volume of the complexes. This additional volume possibly showing the shape of bound Salp15 can be seen in the overlays shown in Fig. 7 (A and B) obtained by manually aligning the models of the complexes with those solved for the unliganded receptor. Alternative superimpositions are also very close and will not change the conclusions drawn, but they do not leave a volume with similar geometric profile unoccupied when comparing the two complexes and unliganded sCD4.

The models shown in Figs. 6 and 7 imply that there is an “end to end” binding between Salp15 and sCD4 molecules. Because the D1 domain is common between the two forms of sCD4 used, it is very likely that Salp15 binds to one side of the apex of the D1 domain. Interestingly, the smaller lobe seen in the unliganded Salp15 was not evident in these models of the Salp15-sCD4 complexes. If the smaller lobe represents the V5/His tag, then its absence in the models of complexes solved for the unliganded receptor. Alternative superimpositions are also very close and will not change the conclusions drawn, but they do not leave a volume with similar geometric profile unoccupied when comparing the two complexes and unliganded sCD4.

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The comparison of the shape profile of the uniform density models restored for the 1:1 Salp15-sCD4 complexes with those generated for unliganded sCD4 molecules suggests that Salp15 binding to the apex of the D1 domain induces a structural rearrangement throughout the long axis of the receptor (Fig. 7, A and B). Upon aligning the Salp15 portion of the complexes in the models, the additional volume in the larger complex shows the shape of the D3–D4 domains in the bigger complex (Fig. 7C). Overall, our models conclude that Salp15 binding leads to a clear loss of native-like bilobal and tetrablobal architecture of unliganded sCD4D1–D2 and sCD4D1–D4, respectively, but the receptor remains semi-extended.

Salp15-sCD4 Interaction Affects the Association of Lck with the Intracellular Portion of CD4 in Activating T Cells—Critical for T cell development and activation, the receptors CD4 and CD8 associate via their cytoplasmic region with the N terminus of the Src family tyrosine kinase Lck (28). Using NMR experiments, it has been shown that in solution the cytoplasmic domain of CD4 and the N terminus of Lck are unstructured in isolation, but they assemble in presence of \( \text{Zn}^{2+} \) to form a compactly folded heterodimeric complex (Protein Data Bank code 1Q68) (28). To examine whether Salp15-induced alteration in the soluble domains of CD4 can eventually affect the ability of the co-receptor to interact with the kinase, we performed immunoprecipitation experiments using unstimulated and anti-CD3/CD28-stimulated purified murine CD4+ T cells in the presence of Salp15.

The association of Salp15 with CD4 results in the improper activation of the Src kinase, Lck during the activation of CD4+ T cells (4). Our results showed that the association of Salp15 and CD4 did not affect the interaction of the co-receptor with Lck in unstimulated cells (Fig. 7D, top panel). The stimulation of the cells with anti-CD3/CD28 resulted in an increased association between CD4 and Lck, which was abrogated by the presence of Salp15 (Fig. 7D). These results showed that the binding of the tick salivary protein and the co-receptor results in the inhibition of the interaction between CD4 and the Src kinase.

In summary, these results showed that Salp15 binding to the D1 domain of sCD4 induces a loss of the distinct domain architecture of the sCD4 receptor without appreciably altering its semi-extended dimensions. These results also raised the question of whether two different ligands that bind to the same region of sCD4 could induce similar conformational changes in the receptor. By comparing the SAXS data-based models of unliganded full-length gp120 and its 1:1 complex with sCD4D1–D2, we have shown that HIV-1 gp120 binding induces shape changes predominantly in the gp120 portion of the complex (15). To determine whether analogous to Salp15, gp120 binding to the apex of sCD4D1–D4 also leaves the full-length sCD4 semi-extended, we carried out SAXS data analysis and molecular modeling of unliganded gp120 and its 1:1 complex with sCD4D1–D4.

1:1 Complex of HIV-1 gp120-sCD4D1–D4—A globular monodisperse nature of the molecules in solution is evident from the SAXS intensity profile collected on the mixture of gp120 and sCD4D1–D4 (Fig. 8A). The slope of the linear fit in the Guinier plot over a \( q \) range of 0.009–0.024 Å\(^{-1}\) indicates that the predominant particle size in gp120-sCD4D1–D4 mixture has an \( R_g \)
of 49.2 ± 0.1 Å (Fig. 8A, inset). $P(r)$ analysis of the SAXS data of the mixture over a $q$ range of 0.01–0.25 Å$^{-1}$ indicated that the scattering data originated predominantly from molecules with a $D_{\text{max}}$ and $R_g$ value of 181 and 49.3 ± 0.1 Å, respectively (Fig. 8B and Table 1). The observed $I_0$ value of 115.2 ± 0.2, indicated almost complete (≈98%) binding between the gp120 and sCD4$_{D1-D4}$ molecules in solution. The contribution to total scattering from any excess sCD4$_{D1-D4}$ would be negligible. Thus, the structural parameters deduced from the SAXS data analysis acquired on this mixture are dominated by the 1:1 complex between these two molecules.

Despite the fact that the sCD4$_{D1-D4}$ is ~65 Å longer than the D1–D2 construct, the $D_{\text{max}}$ of the gp120:sCD4$_{D1-D4}$ complex as shown in Fig. 8B is merely ~15 Å longer in $D_{\text{max}}$ than the corresponding complex with shorter D1–D2 ($D_{\text{max}}$ values of CD4$_{D1-D2} = 75$ Å; HIV-1 gp120 = 166 Å; and gp120:sCD4$_{D1-D2} = 165$ Å) (Table 1) (15). If D1 is the only domain in contact with gp120, and the D1–D4 domains remain semi-extended as seen in the unliganded form, then one would expect the $D_{\text{max}}$ to be greater than 220 Å. This surprising disparity between the expected and the observed dimension of the complex suggests a significant structural change not seen previously from complexes lacking D3–D4 domains.

The average uniform density model of the gp120:sCD4$_{D1-D4}$ complex calculated using the constraints within the measured SAXS data is shown in Fig. 9 (A and B). Previous structural data concluded from various crystallographic studies of the gp120 core bound to sCD4$_{D1-D2}$ and our SAXS data based modeling have shown that the bilobal arm of sCD4$_{D1-D2}$ extends below the volume of bound gp120 in a manner comparable with its unliganded form (15). Surprisingly, the SAXS-based model solved for the gp120:sCD4$_{D1-D4}$ complex shows no features that could accommodate the semi-extended shape seen for unliganded sCD4. This shape change in the CD4 receptor was in complete contrast to what is deduced from the complexes of gp120:sCD4$_{D1-D2}$ as well as from that observed for the Salp15 bound to sCD4$_{D1-D4}$.

To identify the volumes corresponding to the D3 and D4 domains of sCD4, we compared the models solved for the gp120 bound to sCD4$_{D1-D2}$ and sCD4$_{D1-D4}$ (Fig. 9, B and C). Although the height of the gp120:sCD4$_{D1-D4}$ complex is also 165 Å as in the gp120:sCD4$_{D1-D2}$, its diagonal dimension is closer to the expected $D_{\text{max}}$ of 180 Å. Comparable orientations of both complexes, rotated along the long axis, clearly show that the smaller complex has less width in the lower portion of the model. Interestingly, a short axis dimension of 25 Å is comparable with the cross-sectional diameter of either domain of sCD4$_{D1-D2}$ as seen in its crystal structures as well as in our SAXS-based model of the two domain protein. Although there are some differences in the upper portion of the complexes very likely residing the bound gp120, we manually aligned the two complexes manually to appreciate the additional volume in one end of the gp120:sCD4$_{D1-D4}$ complex (Fig. 10A). Earlier, in the chain ensemble model of the gp120:sCD4$_{D1-D2}$ complex, we used the shape profile of the bilobal projection in the molecule to fit the crystal structure of gp120 core bound to sCD4$_{D1-D2}$ (15).

Similarly, using the shape profile of the bilobal arm of CD4 extending below the volume of gp120 in the complex and the coordinates of CD4$_{D1-D2}$ molecule in the crystal structure 2B4C (11), we placed the structure of gp120 core-CD4$_{D1-D2}$ inside our SAXS-based model (Fig. 10B). This alignment aided
FIGURE 10. HIV-1 gp120 binding induces a bi-fold closure of full-length sCD4 molecule. A, alignment of the models solved for gp120/sCD4D1–D4 and gp120/sCD4D1–D2 in three-dimensional space show unoccupied volume on one end of the purple complex. B, the volumes of gp120 core bound to sCD4D1–D2 from crystal structure Protein Data Bank code 2B4C are employed to confirm the volumes corresponding to bound gp120 and sCD4 in our models of the complexes. Placement of the crystal structure inside overlaid models of complexes confirms that the D3–D4 domains in full-length sCD4 are folded next to D1–D2 domains bound to gp120.
A proposal that the D2–D3 junction can bend substantially without affecting the structure of the D1–D2 and the D3–D4 halves is supported by the following observations: 1) using the sCD4 D1–D4 crystal structure, large movements across this hinge leading to acute angles can be modeled without significant steric hindrance (2); 2) limited proteolysis studies on intact human sCD4 D1–D4, with V8 and chymotrypsin, results in only two stable and functionally active products identified to be the D1–D2 and the D3–D4 halves (26); and 3) except for Arg^59, which lies protected inside the binding pocket, no change in solvent exposure of Arg residues was observed before versus after binding to gp120 (29).

Further evidence of the importance of this junctional flexibility can be seen in the literature. Huang et al. (25) showed that almost all of the mutations in the hinge region of sCD4 (alanine scan from 175–181 and, deletion mutants DEL 175–177 and DEL 180–181) showed substantial loss of interleukin-2 production compared with wild-type CD4-expressing cells. Among the mutants screened, the deletion of residues 175–177 indicated maximum loss of structural information present in the native protein because it could not be recognized by 14 of the 18 monoclonal antibodies of native sCD4. Also, the binding of monoclonal antibody Q425 near the D2–D3 junction was shown to block HIV-mediated membrane fusion without affecting gp120 binding to the D1 domain of sCD4 (30, 31). On an analogous level, the flexibility in the linker region connecting the D4 domain and the transmembrane helix of CD4 is considered important for immune responses (32).

**Physiological Relevance of Salp15- or gp120-induced Specific Shape Changes in Their Receptor sCD4**—Clear differences observed in the shape of the complexes solved for Salp15=sCD4 and gp120=sCD4 confirm that ligand binding on the same region of the receptor CD4 can induce very different and specific shape changes in the receptor. Based on known follow-up biological steps, it appears that both ligands induce the observed structural reorganization in CD4 to achieve their intended function.

It has been shown that by binding to the D1 domain of the T cell receptor CD4 and repressing the activation of Lck, Salp15 triggers the inhibition of T cell activation (4). Our models show that the Salp15 monomer forms a 1:1 complex with the CD4 molecule and distorts the native geometry of the receptor (Fig. 11A). In comparison with the models solved for the unliganded sCD4 molecules, Salp15 binding leads to the loss of interdomain shape of the receptor. This stage in conjunction with the results obtained from co-immunoprecipitation experiments...
helps in delineating the mechanism of action of Salp15 in T cells.

In contrast, a bi-fold collapse of soluble domains of sCD4 induced by 1:1 binding of gp120 molecule correlates with the need to initiate a different series of events than that seen with Salp15. It is well known that structural reorganization within the bound gp120 exposes its immunogenic V3 loop, which subsequently binds to extracellular segments of the co-receptors CCR5 or CXCR4 leading to fusion of viral and host cell membranes and eventually, viral entry (33). Lipid raft localization experiments have shown that sCD4 mutants lacking D3–D4 domains are unable to facilitate viral entry, highlighting the need to study gp120 binding to the entire four domain structure of sCD4 (34). This observed collapse of sCD4 would facilitate positioning of the exposed V3-loop of bound gp120 closer to the other extracellular loops of CCR5 or CXCR4 (Fig. 11B). Because the immunogenic V3 loop of CD4-bound gp120 can stretch out only ~35 Å in space (11) and the relatively short (13–30 residues) extracellular segments of the co-receptor are anchored close to the membrane (33), an extended structure of sCD4 would prevent the communication between the V3 loop of bound gp120 and thus must be required for membrane fusion without affecting gp120 binding to the D1 domain of CD4 (30, 31). These results provide the first confirmation that structural rearrangement within CD4 ectodomains occurs upon binding gp120 and thus must be required for HIV-1 viral entry.

The results presented here confirm that the ligand-induced shape changes in receptor sCD4 are specific and have a clear correlation with the follow-up biological events. Our experiments with full-length sCD4 containing all four domains and glycosylation demonstrate the important role played by the D3–D4 domains and especially the functional flexibility between the domains of the CD4 molecule.

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