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Journal of Immunological Methods 210 (1997) 215–225

JOURNAL OF IMMUNOLOGICAL METHODS

Construction and characterization of a radio-iodinatable mutant of recombinant human CD4

Marc Ferrer [a], Kevin L. Godbout [c], Barbara J. Sullivan [c], Douglas A. Austen [c], Christine T. Sanderson [c], Kevin C. Kelley [c], Marcia S. Osburne [c], Stephen C. Harrison [a,b], Marie R. van Schravendijk [c,*]

[a] *Department of Molecular and Cellular Biology, Harvard University, 7 Divinity Ave, Cambridge, MA 02138, USA*
[b] *Howard Hughes Medical Institute, Harvard University, 7 Divinity Ave, Cambridge, MA 02138, USA*
[c] *Procept Inc., 840 Memorial Dr., Cambridge, MA 02139, USA*

Received 3 September 1997; revised 4 November 1997; accepted 4 November 1997

Abstract

Recombinant soluble human CD4 (rsCD4) has been used in iodinated form to study the interaction of CD4 with its ligands. However, the utility of [^{125}I]-rsCD4 is limited because rsCD4 is inefficiently iodinated and the iodinated protein is poorly active. The iodination properties of rsCD4 most likely reflect the poor accessibility of the tyrosine residues, apparent from the available X-ray structures. We have generated an iodinatable mutant of rsCD4 by substituting Tyr for Phe$_{179}$ in the flexible, solvent-exposed C-terminal region of rsCD4$_{183}$, a truncated form of CD4 that consists of the first 183 residues of CD4 and includes the binding sites for HIV-1 gp120 and MHC class II molecules. When F179Y rsCD4$_{183}$ is iodinated under trace-labeling conditions, the efficiency of ^{125}I incorporation and the percentage of iodinated molecules that are active are much enhanced compared with WT rsCD4. Moreover, trace-labeled [^{125}I]-F179Y rsCD4$_{183}$ has the same affinity for HIV-1 rgp120 as unlabeled WT rsCD4. The improved activity of trace-labeled [^{125}I]-F179Y rsCD4$_{183}$ appears to be due to effective competition by Y179 for reactive iodine species that, in WT rsCD4, react with traces of denatured protein and/or with residues critical for activity or conformational integrity. The incorporation of accessible tyrosine residues may improve the iodinatibility of a protein both by introducing a readily iodinatable residue and by protecting sensitive proteins from adverse reactions.

Keywords: CD4; HIV-1; gp120; Purification; Iodination; Radioimmunoassay

1. Introduction

CD4 is a 55 kDa transmembrane glycoprotein with an extracellular region consisting of four immunoglobulin-like domains D1–D4 (reviewed in Littman, 1995). CD4 present on CD4$^+$ T-cells functions as a co-receptor specific for MHC class II

Abbreviations: AEBSF, 4-(2-aminoethyl)benzene sulfonylfluoride; BSA, bovine serum albumin; D1–D4, domains 1 through 4 of CD4; DTT, dithiothreitol; MWCO, molecular weight cutoff; PBS, 20 mM potassium phosphate pH 7.4, containing 130 mM NaCl; PBST, PBS containing 0.05% w/v Tween-20; PBSTB, PBST containing 10 mg/ml BSA; WT, wild type

* Corresponding author. ARIAD Pharmaceuticals, 26 Landsdowne St., Cambridge, MA 02139, USA. Tel.: +1-617-4940400 ext. 362; fax: +1-617-4948144; e-mail: vanschra@ariad.com

0022-1759/97/$17.00
PII S0022-1759(97)00195-6

molecules during antigen-driven T-cell activation. In T-cell activation, binding of CD4 to MHC class II molecules requires formation of an oligomeric cell surface complex in which D1D2 of CD4 interact with MHC class II molecules and D3D4 of CD4 participate in formation of CD4 oligomers (Konig et al., 1995; Sakihama et al., 1995; Wu et al., 1997). CD4 present on $CD4^+$ T-cells also acts as a receptor for human immunodeficiency virus type 1 (HIV-1), which attaches to CD4 through its envelope glycoprotein gp120 (reviewed in Littman, 1995). Binding of CD4 to gp120 is thought to induce a conformational change in gp120 which leads to virus–host cell fusion and viral entry, a process which requires the interaction of gp120 with specific chemokine receptors on the host cell surface (reviewed in Broder and Dimitrov, 1996). Binding of cell-surface CD4 to HIV-1 gp120 is of high affinity, ranging from 1 to 30 nM for different variants of gp120 (Ivey-Hoyle et al., 1991; Moebius et al., 1992b), and involves only CD4 D1, as shown using soluble rsCD4 constructs (Arthos et al., 1989). Site-directed mutagenesis studies have shown that the C′C″ loop region of CD4 D1 is the primary binding site for gp120 and that residues K35, F43, K46 and R59 are particularly important for this interaction. These residues of the C′C″ region are also involved in binding of MHC class II, although the binding site is more extensive than that of gp120 (Moebius et al., 1992a,b, 1993; Houlgatte et al., 1994).

For molecular studies of the interactions of CD4 with its natural ligands or with small molecules designed to block those interactions, it would be desirable to have radiolabeled rsCD4 of high specific activity that is fully active. Site-specific labeling of the carbohydrates in rsCD4 D3 with [^{3}H] has been achieved (Terhorst et al., 1980; Chamow et al., 1992), but is applicable only to CD4 molecules carrying sialic acid. Given the importance of lysine residues in the binding sites for gp120 and MHC class II, labeling of lysine residues is undesirable, and indeed modification of rsCD4 with a succinimide-based reagent has been shown to result in impaired activity (Chamow et al., 1992). CD4 has been iodinated by means of lysine-directed reagents such as the Bolton–Hunter reagent (Portoles et al., 1990; Ivey-Hoyle et al., 1991). However, 50% of rsCD4 iodinated with the Bolton–Hunter reagent was inactive in immunoprecipitation with gp120 (Ivey-Hoyle et al., 1991). Human CD4 is not readily iodinated by other methods (Sayre and Reinherz, 1985). In general, iodinated CD4 has been used under conditions where less than 20% is bound to its ligand, so that the degree of inactivation cannot be evaluated (Arthos et al., 1989; Fenouillet et al., 1989; Davis et al., 1992; Autiero et al., 1995).

We engineered a more readily iodinatable form of human CD4 by substituting tyrosine for phenylalanine 179 in the C-terminal region of a truncated form of CD4, $rsCD4_{183}$. $rsCD4_{183}$ contains the N-terminal 183 residues, which form the first 2 domains (D1D2). The F179Y mutation is distal to the gp120 and MHC-binding sites and the structure of D1D2 shows that it is in a flexible, solvent-exposed region, likely to be readily accessible for labeling. The engineered CD4 protein incorporated iodine more readily than WT CD4 and the labeled form was more active than the WT iodinated protein. Our studies suggest that the improved labeling and activity of F179Y $rsCD4_{183}$ result from effective competition by Y179 for active iodine species that participate in undesirable reactions with the WT protein. Therefore, the judicious incorporation of tyrosine residues into a protein is likely to be useful for other proteins that are poorly active upon iodination.

2. Materials and methods

2.1. Expression of rsCD4

The hu $rsCD4_{183}$ coding sequence was cloned and expressed in *E. coli* strain BL21 (DE3) as described (Kelley et al., 1995), using vector pET-9a (Novagen, Madison, WI). Cells were grown without induction in 2 × YT medium at 37°C, resulting in high-level production of rsCD4 (Kelley et al., 1995). The F179Y $rsCD4_{183}$ nucleotide sequence was constructed by PCR mutagenesis and expressed in vector pHN1 (MacFerrin et al., 1990) as follows. An oligonucleotide encoding the *Eco*RI restriction site and an ATG start codon (CGCGCGAATTCAGGAGGAATTTAAAATGAGGAAAGTGGTGC), and an oligonucleotide encoding the *Hin*dIII restriction site and the mutant F179Y (TTC to TAC) mutant codon (GCGCGCAAGCTTTTAGGAGGCCTTCTG-

GTAAGCTAG) were used as the 5′ and 3′ PCR primers, respectively. The PCR was carried out using the WT rsCD4$_{183}$ nucleotide sequence as the template. The mutant sequence was cloned into vector pHN1, utilizing the added restriction sites, and the plasmid was transformed into *E. coli* strain XA 90 F′ *lacI*^{Q} (MacFerrin et al., 1990). The resulting clones were screened for rsCD4 production by SDS-PAGE analysis of whole cell protein (Kelley et al., 1995). For production of rsCD4$_{183}$ F179Y protein, cells were grown in a 5 l fermentor vessel (B. Braun Biotech, Melsungen, Germany) at 37°C in 2 × YT medium to $OD_{600} = 0.8$, induced with 0.5 mM β-D-thiogalactopyranoside (IPTG) and harvested 16–18 h later.

The hu rsCD4$_{371}$ cDNA was derived from the four-domain cDNA sequence encoding the soluble portion of CD4 (Maddon et al., 1985). A 5′ *Bam*H1 site and a 3′ *Eco*R1 site were engineered onto the sequence by PCR and the amplified DNA was then cloned into those sites in the baculovirus expression vector pVL 1393 (Pharmingen, San Diego, CA). The recombinant vector was then transfected into Sf9 insect cells using the BaculoGold™ transfection system (Pharmingen) to select for recombinant baculovirus. For production runs, Sf9 cells were grown at 28°C in serum-free medium (SF 900 II medium, Gibco BRL, Gaithersburg, MD) in 8-l spinner flasks (Bellco Glass, Vineland, NJ) and infected with recombinant baculovirus at a multiplicity of infection of 5. Cell supernatant was harvested 72 h post infection.

2.2. Purification of rsCD4$_{183}$

Tergitol-treated inclusion bodies (Garlick et al., 1990) were washed with 50 mM sodium phosphate buffer pH 7.0 containing 1 M guanidine hydrochloride and 1 mM EDTA, and dissolved in 50 mM sodium phosphate buffer pH 7.0, containing 6 M guanidine hydrochloride and 1 mM EDTA, at a protein concentration of 10–20 mg/ml as determined by a Coomassie-based protein assay (BioRad Laboratories, Richmond CA). The extract was clarified by centrifugation at 10,000 *g* for 30 min and reduced with 4 mM DTT at room temperature for 1.5 h. The protein was refolded at 4°C for 16 h by dilution to 0.1 mg/ml protein in 0.1 M Tris/Tris–HCl buffer pH 8.0, containing 1 mM EDTA, 0.3 mM oxidized glutathione, 3 mM reduced glutathione and a final guanidine hydrochloride concentration of 0.2 M. Refolded material was treated with 0.1 mM of the protease inhibitor AEBSF (Calbiochem, San Diego, CA), exchanged into PBS using a 5,000 MWCO Pellicon membrane (Millipore, Bedford, MA) and purified by immunoaffinity chromatography on anti-CD4 mAb 19Thy5D7 (Moebius et al., 1992b, 1993) coupled to Fast-Flow Immobilized rProtein A (Repligen Corp, Cambridge, MA) at a concentration of 7 mg mAb/ml resin as described (Harlow and Lane, 1988). rsCD4$_{183}$ was eluted with 50 mM citric acid/sodium citrate buffer pH 3.0, neutralized, treated with 0.1 mM AEBSF and further purified on a 2.6 × 110 cm Sephacryl S100HR column (Pharmacia Biotech, Piscataway, NJ) in 50 mM HEPES pH 7.9. The purified rsCD4 was stored at −70°C at a concentration of 5–10 mg/ml.

2.3. Other reagents

Anti-CD4 D1 mAb Leu3a was obtained from Becton-Dickinson (San Jose CA). Anti-CD4 D1 mAbs 19Thy5D7 (mouse IgG2a) and 18T3A9 (mouse IgG1) (Moebius et al., 1992b, 1993) and Procept anti-gp120 mAb 803-15.6 (mouse IgG1) directed against the C-terminal peptide of gp120 (K.L.G., unpublished results) were purified from serum-free culture supernatant on protein A- or protein G-Sepharose (Pharmacia Biotech). mAbs 18T3A9 and 803-15.6 were coupled to Fast-Flow Immobilized rProtein A at 2 mg mAb/ml resin as described (Harlow and Lane, 1988). rsCD4$_{371}$ produced in the baculovirus system was purified by affinity chromatography on mAb 19Thy5D7-coupled affinity resin. Traces of immunoglobulin were removed by absorption with protein A- and protein G-Sepharose. HXB2 gp120 cDNA was the gift of Dr. Dan Littman (Page et al., 1990). HXB2 gp120 produced in the baculovirus system using serum-free medium (SF900 II medium, Gibco BRL) was purified by affinity chromatography on mAb 803-15.6-coupled affinity resin followed by rsCD4$_{183}$-Sepharose.

2.4. Protein characterization

The purity and structure of purified rsCD4 was analyzed by reduced and non-reduced SDS-PAGE,

size exclusion HPLC on a 7.5×600 mm TSK G2000SW column (10 μm), electrospray mass spectrometry on a Micromass Platform II (Micromass, Beverly, MA), and amino acid analysis. The conformation of purified rsCD4 was analyzed by competition ELISA with gp120 and mAbs that recognize conformational epitopes on CD4. For competition ELISA, $rsCD4_{371}$ (0.1 ml, 1 μg/ml in PBS) was coated on Immulon-2 plates (Dynatech Inc, Chantilly, VA) for 16 h at 4°C. Control wells were coated with PBS alone. The plate was blocked for 2 h at 4°C with 0.2 ml PBSTB. All ELISAs were performed in a total volume of 0.2 ml PBSTB per well. Anti-CD4 mAbs (5 ng/ml) or HXB2 gp120 (15 ng/ml) and soluble CD4 samples (0–100 nM) were added to the $rsCD4_{371}$-coated plate and incubated for 16 h at 4°C. After washing five times with PBST, bound gp120 was probed by incubation with 200 ng/ml anti-gp120 mAb NEA9205 (NEN Dupont, Boston, MA) for 2 h at 4°C. Bound mAbs were detected by incubation with alkaline phosphatase-labelled secondary antibodies (Southern Biotech, Birmingham, AL). Protein concentrations were determined by A_{280} using the following $\varepsilon_{280}^{(1\ mg/ml,\ 1\ cm)}$: 0.90 for WT $rsCD4_{183}$, 0.95 for F179Y $rsCD4_{183}$, 1.54 for $rsCD4_{371}$ and 0.62 for HXB2 gp120.

2.5. Iodination

Iodination was carried out using the Iodogen method (reviewed in Parker, 1990). For standard trace-labeling with ^{125}I, 5 μg protein in 50 μl 50 mM sodium phosphate buffer pH 7.4, containing 100 mM NaCl (final concentration 5 μM for $rsCD4_{183}$, 2.5 μM for $rsCD4_{371}$) were iodinated in a tube coated with iodogen (Pierce Chemical Co.) with 50 μCi [^{125}I]Na (2,100 Ci/mmol, final concentration 0.5 μM carrier free ^{125}I) (Amersham Corp., Arlington Hts, IL) for 10 min at room temperature. The best results were obtained by centrifuging rsCD4 before iodination and omitting agitation of the iodination tube, as rsCD4 is sensitive to shear. Following iodination, [^{125}I]-labeled rsCD4 was separated from free ^{125}I using a PD10 column (Pharmacia Biotech, Piscataway, NJ) equilibrated with PBS containing 0.1% Tween-20 (Sigma Chemicals Co., St. Louis, MO) and 0.05% sodium azide. [^{125}I]-labeled protein was recovered in a volume of 1.6 ml. Iodinated samples were counted on a Micromedic Systems 28036 gammacounter (ICN, Costa Mesa CA) with a counting efficiency of 76%. For measurement of protein recovery in mock iodinations, [^{125}I]-labeled rsCD4 was diluted 50-fold into 0.1 mg/ml unlabeled rsCD4 and treated as above except that no ^{125}I was added. γ-Glu–Tyr (Sigma Chemical Co., St. Louis, MO) ($\varepsilon_{280} = 1{,}200\ M^{-1}\ cm^{-1}$) was used as a 3 mM stock solution in water.

2.6. Immunoprecipitation of [^{125}I]-labeled rsCD4

For immunoprecipitation assays determining the maximal binding of [^{125}I]-labeled rsCD4 to mAbs and gp120, [^{125}I]-labeled rsCD4 was added to a total volume of 1 ml PBSTB containing 10 μl packed affinity resin (coupled with 19Thy5D7, 18T3A9, or 803-15.6). In the case of mAb 803-15.6 resin, 17 nM HXB2 gp120 was added concurrently with rsCD4. The samples were rotated end-over-end for 3 h at room temperature, which was shown to be sufficient for full equilibration. To determine the affinity of [^{125}I]-labeled rsCD4 and gp120, a titration of 0–10 nM [^{125}I]-labeled and unlabeled rsCD4 was used with a constant amount of HXB2 gp120 (0.75 nM) and 803-15.6 affinity resin (5 μl) and an incubation time of 5 h. After incubation, samples were centrifuged for 2 min at 2,000g and half of the supernatant (0.5 ml) was withdrawn (0.5S). The cpm in this supernatant and in the remaining contents of the reaction tube (R), representing the pellet and the remaining half of the supernatant, were counted. The fraction of total cpm that bound to the resin was calculated as $(R - 0.5S)/(R + 0.5S)$. Controls without beads, with protein A resin alone, or with mAb 803-15.6-coupled affinity resin alone showed no detectable non-specific binding of [^{125}I]-labeled rsCD4.

3. Results

3.1. Purification and characterization of WT and F179Y hu $rsCD4_{183}$

Both WT and F179Y $rsCD4_{183}$ were produced in *E. coli* as inclusion bodies. The yields were 0.87 ± 0.12 mg/g cell paste for WT $rsCD4_{183}$ (7 experiments) and 0.40 ± 0.08 mg/g cell paste for F179Y

Table 1
Competition ELISA analysis of unlabeled rsCD4

Competition ELISA	IC50/nM		
	F179Y $rsCD4_{183}$	WT rs $CD4_{183}$	$rsCD4_{371}$
mAb 18T3A9	5.8 ± 0.8 [8]	5.5 ± 1.0 [6]	6.4 ± 0.9 [6]
mAb 19Thy5D7	5.0 ± 0.4 [6]	4.6 ± 0.8 [4]	4.1 ± 0.8 [4]
mAb Leu3a	0.13 ± 0.01 [6]	0.16 ± 0.02 [4]	0.19 ± 0.03 [4]
HXB2 rgp120	0.65 ± 0.09 [6]	0.66 ± 0.12 [4]	0.80 ± 0.10 [4]

For all competition ELISA's, $rsCD4_{371}$ was coated in the wells at 1 μg/ml. The mAb indicated, or gp120 was added for 16 h at 4°C, in the absence or presence of the different types of CD4 in solution. The IC50 of the solution phase CD4 was determined as the concentration resulting in 50% decrease in signal relative to the signal in the absence of CD4. The mean IC50 and standard error of the mean for each type of CD4 are shown, with the number of assays in square brackets.

$rsCD4_{183}$ (2 experiments). The final proteins were pure as determined by SDS-PAGE and native size exclusion HPLC (data not shown). Comparative amino acid analysis (data not shown) was consistent with the presence of the human CD4 sequence plus the expected N-terminal methionine (Ben-Bassat and Bauer, 1987) and with the replacement of one phenylalanine with a tyrosine residue in F179Y $rsCD4_{183}$. The molecular mass values obtained by electrospray mass spectrometry were $(20{,}388 \pm 2)$ for WT and $(20{,}404 \pm 2)$ for F179Y $rsCD4_{183}$, which agrees with the theoretical values (20,389 for WT and 20,405 for F179Y). Correct refolding of WT $rsCD4_{183}$ was confirmed by crystallization in the same crystal form and under the same conditions as described previously (Wang et al., 1990; Wang, personal communication). Furthermore, WT and F179Y $rsCD4_{183}$ proteins refolded from *E. coli* inclusion bodies had identical activity to baculovirus-produced $rsCD4_{371}$ in four different competition ELISAs with HIV-1 rgp120 and mAbs that bind to conformational epitopes on D1 of CD4 (Table 1). Both F179Y $rsCD4_{183}$ and WT $rsCD4_{183}$ could be stored at 4°C for at least 2 weeks or frozen at -70°C for 2 years without loss of activity.

3.2. Iodination of rsCD4

Under the standard trace-labeling conditions, with a molar ^{125}I:protein ratio of 1:10, an average of 40% of input ^{125}I was incorporated into F179Y $rsCD4_{183}$ (Table 2). In two experiments where F179Y $rsCD4_{183}$ was iodinated in parallel with purified mAb 19Thy5D7, the level of iodination achieved was 100 ± 10% of that of the immunoglobulin. By contrast, WT $rsCD4_{183}$ and $rsCD4_{371}$ consistently gave 2–3-fold lower incorporation of ^{125}I (Table 2).

The iodination of the $rsCD4_{183}$ species was characterized in three ways. First, to quantitate iodine incorporation on a molar basis, several control experiments were performed. (i) Analysis by SDS-PAGE followed by autoradiography showed that the radiolabel co-migrated with the rsCD4 band (data not shown). (ii) Mock iodinations using 50 μCi ^{125}I but no protein, showed that the amount of soluble ^{125}I recovered in the rsCD4 fraction was 0.9 ± 0.2% of the soluble cpm loaded ($n = 5$). (iii) Mock iodinations using [^{125}I]-$rsCD4_{183}$ as a tracer, but no soluble ^{125}I, showed that protein recovery for both F179Y and WT $rsCD4_{183}$ was 46 ± 4% ($n = 11$). From these control experiments, it can be calculated that incorporation of 22 μCi ^{125}I into F179Y and 10 μCi

Table 2
Iodination of rsCD4

Iodination contion	[^{125}I]incorporation/μCi		
	F179Y $rsCD4_{183}$	WT $rsCD4_{183}$	$rsCD4_{371}$
50 μCi ^{125}I	22.3 ± 2.0 [13]	10.0 ± 1.2 [9]	8.3 ± 1.4 [2]
50 μCi ^{125}I, 1 μM γ-glu–tyr	15.6 ± 4.1 [2]	2.2 ± 0.2 [2]	
200 μCi ^{125}I		26.9 [1]	
50μCi ^{125}I (24 pmol) + 2 nmol unlabeled iodide	2.0 [1]	2.8 ± 0.1 [2]	

The table shows μCi ^{125}I incorporated in experiments under the indicated conditions. All experiments used iodogen-coated tubes and 5 μg CD4 in a volume of 50 μl 50 mM potassium phosphate buffer, pH 7.2, containing 100 mM NaCl. Data are shown as mean $\pm$ standard error of the mean, with the number of experiments in square brackets. Data have not been corrected for soluble ^{125}I cpm carried through the desalting column, which was (0.9 ± 0.2) % of the soluble cpm loaded.

Table 3
Fraction of iodinated rsCD4 active in immunoprecipitation with mAb 19Thy5D7

CD4 sample	F179Y $rsCD4_{183}$	WT $rsCD4_{183}$	$rsCD4_{371}$
Labeled with 50 μCi [^{125}I]	70 ± 5 [6]	28 ± 3 [4]	21 ± 5 [2]
Labeled in presence of γ-Glu–Tyr	74 ± 3 [2]	36 ± 2 [2]	
Labeled with excess iodide	10 [1]	13 [1]	

The table shows the % of labeled molecules able to bind to 19Thy5D7 in the immunoprecipitation assay. Immunoprecipitation was carried out with 10 μl 19Thy5D7-coupled affinity resin in a total volme of 1 ml, rotated at room temperature for 3 h. Under these conditions, F179Y $rsCD4_{183}$ is maximally bound. Data have been corrected for the small amount of soluble cpm in the labeled preparations.

into WT $rsCD4_{183}$ (Table 2) represents approximately 0.09 mol I/mol CD4 for F179Y and 0.04 mol I/mol CD4 for WT.

Second, to determine if 0.04 mol I/mol was the maximum incorporation level for WT $rsCD4_{183}$ (such as would be the case if it were incorporated into a small fraction of denatured protein), the amount of iodide present during iodination was increased. Incorporation of ^{125}I was increased with increasing iodide (Table 2). In the presence of an eight-fold molar excess of unlabeled iodide, total iodide incorporation was in the range 0.5–1 mol I/mol CD4 for both WT and F179Y $rsCD4_{183}$.

Third, the nature of the labeling reaction in WT $rsCD4_{183}$ was probed by adding soluble γ-Glu–Tyr as a competitor. The presence of 1 μM γ-Glu–Tyr strongly depressed the level of iodination of WT $rsCD4_{183}$, with less effect on F179Y (Table 2).

3.3. Activity of iodinated rsCD4

To characterize the effect of iodination on the activity of the recombinant CD4 proteins, several different methods were used. First, the fraction of [^{125}I]-labeled rsCD4 molecules in each preparation that was able to bind to excess mAb 19Thy5D7-coupled affinity resin was determined in immunoprecipitation assays. 70% of [^{125}I]-labeled F179Y $rsCD4_{183}$ molecules were able to bind to 19Thy5D7, as compared with only 28% of [^{125}I]-labeled WT $rsCD4_{183}$ and 21% of [^{125}I]-labeled $rsCD4_{371}$ (Table 3).

The nature of the inactive material detected by immunoprecipitation was further investigated by

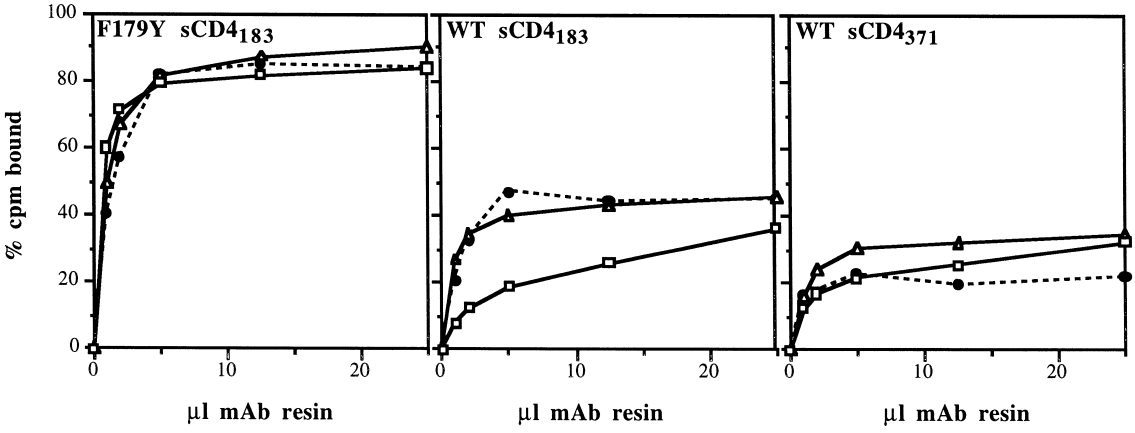

Fig. 1. Immunoprecipitation of [^{125}I]-labeled CD4 constructs with anti-CD4 mAbs. Trace-labeled [^{125}I]-CD4 (10,000–50,000 cpm) was nutated with the indicated volume of affinity resin (coupled with mAb 19Thy5D7, 18T3A9, or 803-15.6) in a total volume of 1 ml PBSTB at room temperature for 3 h. In the case of 803-15.6 affinity resin, 17 nM HXB2 gp120 was also present. The graph shows the % of total cpm bound to the affinity resin. Left panel: F179Y $rsCD4_{183}$; Middle panel WT $rsCD4_{183}$; Right panel: $rsCD4_{371}$. Symbols: □, 19Thy5D7-coupled resin; △, 18T3A9-coupled resin; ●, HXB2 gp120 on 803-15.6-coupled resin.

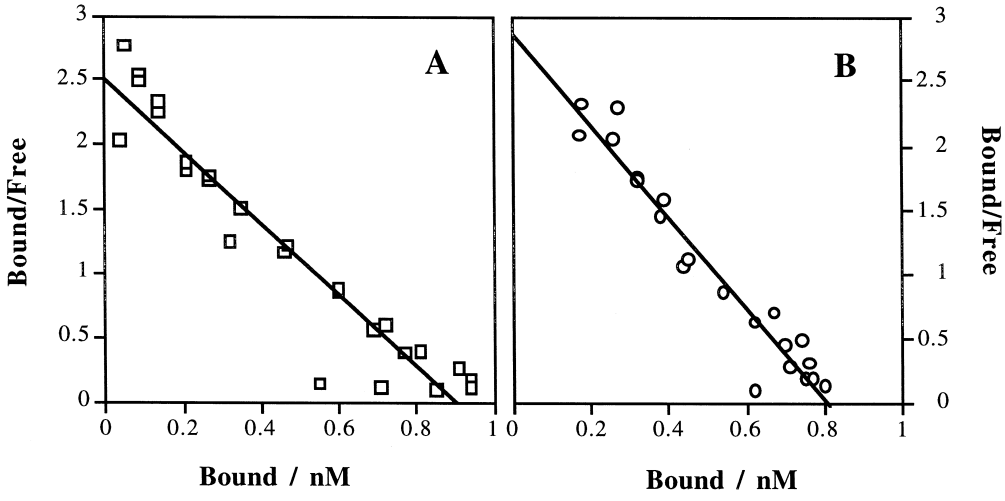

Fig. 2. Scatchard plots of [^{125}I]-labeled CD4 binding to gp120. Trace-labeled [^{125}I]-F179Y rsCD4$_{183}$ was added to a total volume of 1 ml PBSTB containing 5 μl packed affinity resin (coupled with mAb 803-15.6) and 0.75 nM HXB2 gp120. The sample was nutated for 5 h at room temperature. The figure shows one of three experiments with similar results. (A) shows the binding titration of increasing amounts of a [^{125}I]-labeled F179Y rsCD4$_{183}$ preparation alone. (B) shows the binding titration calculated for a constant amount of [^{125}I]-labeled F179Y rsCD4$_{183}$ (0.25 nM) in the presence of 0–7 nM unlabeled WT rsCD4$_{183}$, assuming that the labeled and unlabeled material are equally active. The straight lines were fitted by linear regression.

comparing binding of [^{125}I]-labeled F179Y and WT rsCD4 to 19Thy5D7, 18T3A9 and HXB2 gp120 affinity resins (Fig. 1). These three conformational probes, which bind to different epitopes of CD4, all detected a large fraction of inactive [^{125}I]-labeled material in WT rsCD4$_{183}$ and rsCD4$_{371}$ (Fig. 1).

To determine whether the reduced activity of iodinated WT rsCD4 was specific to the labeled fraction, the activity of the rsCD4 preparations was measured in competition ELISA with mAb 19Thy5D7. This assay measures the overall activity of the total population of rsCD4 molecules (both unlabeled and labeled molecules) relative to an unlabeled rsCD4 standard of known concentration. We found that the ELISA activities of the trace [^{125}I]-labeled F179Y and WT rsCD4 preparations were similar (ratio F179Y:WT = 0.82 ± 0.08 (3 experiments)). Therefore, the unlabeled molecules in the [^{125}I]-labeled WT rsCD4$_{183}$ preparation seemed as active as those in F179Y.

To determine whether the affinity of trace-labeled [^{125}I]-F179Y rsCD4$_{183}$ for gp120 was the same as that of native unlabeled WT rsCD4, binding titrations were performed in the immunoprecipitation format, using mAb 803-15.6 affinity resin. A constant amount of gp120 (0.75 nM) was titrated either with unlabeled WT rsCD4$_{183}$ (0–7 nM) in the presence of a constant amount [^{125}I]-labeled F179Y rsCD4$_{183}$ (0.25 nM), or with [^{125}I]-labeled F179Y rsCD4$_{183}$ alone (0–9 nM). Both titrations gave linear Scatchard plots (Fig. 2) and yielded similar binding parameters in three experiments. For [^{125}I]-labeled F179Y rsCD4$_{183}$ alone, $K_d = 0.35 \pm 0.05$ nM and the number of binding sites per mol gp120 = 1.3 ± 0.1. The competition experiment with unlabeled WT rsCD4$_{183}$ gave $K_d = 0.30 \pm 0.04$ nM and the number of binding sites per mol gp120 = 1.4 ± 0.1.

In contrast to trace-labeling, iodination with an 8-fold molar excess of iodide over rsCD4 caused complete inactivation of both F179Y and WT rsCD4$_{183}$, as assayed both by immunoprecipitation with 19Thy5D7 (Table 3) and competition ELISA with 19Thy5D7 and mAb Leu3a (data not shown).

4. Discussion

Early immunochemical studies showed that human cell surface CD4 is not readily iodinated by lactoperoxidase (Sayre and Reinherz, 1985). This

was attributed to the small number of tyrosine residues in the extracellular portion of human CD4 (Portoles et al., 1990). The three-dimensional structures of D1D2 of human rsCD4 and D3D4 of rat rsCD4 have now been determined at high resolution (Ryu et al., 1990; Wang et al., 1990; Brady et al., 1993), and the structure of human $rsCD4_{369}$ at moderate resolution (Wu et al., 1997). The extracellular region of human CD4 has three tyrosine residues: Y82 in the F strand of D1 (Maddon et al., 1985; Ryu et al., 1990; Wang et al., 1990) and Y187 and Y266 in D3 (Maddon et al., 1985; Brady et al., 1993; Wu et al., 1997). Of these tyrosines, Y82 is the most inaccessible. Although Y187 and Y266 are partially solvent exposed, they are not fully accessible to a probe sphere the size of an iodine atom (radius 2.0 Å) (Wu et al., 1997, PDB accession number 1wio). Our iodination data also suggest that the tyrosines in D3 are not readily available for iodination, as there was no improvement in iodination or activity of WT $rsCD4_{371}$ as compared with WT $rsCD4_{183}$.

To generate a construct of CD4 readily iodinatable at tyrosine, we made a conservative substitution of tyrosine for F179 in the C-terminal region of $rsCD4_{183}$. The crystal structure of WT $rsCD4_{183}$ (Wang et al., 1990; Ryu et al., 1990) shows that the C-terminal region from F179 onward is disordered. A mutation in this position would be expected to have little effect on the conformation of CD4. Our characterization of the unlabelled protein confirms that the conformation of F179Y $rsCD4_{183}$ is very similar to WT $rsCD4_{183}$ (Table 1). The high mobility of F179 apparent from the structural data also suggests that the corresponding tyrosine residue would be freely accessible to iodination. Indeed, F179Y $rsCD4_{183}$ gave consistently 2-3-fold higher iodine incorporation than either WT $rsCD4_{183}$ or $rsCD4_{371}$ (Table 2). In addition, this trace-labeled [^{125}I]-F179Y $rsCD4_{183}$ was consistently much more active than [^{125}I]-WT $rsCD4_{183}$ or $rsCD4_{371}$ (Table 3, Fig. 1). The similar titration results with [^{125}I]-F179Y $rsCD4_{183}$ alone and in competition with unlabeled WT $rsCD4_{183}$ show that [^{125}I]-F179Y $rsCD4_{183}$ had the same affinity for HIV-1 rgp120 as unlabeled WT rsCD4 (Fig. 2).

When iodinating with high iodide to protein ratios, both WT and F179Y $rsCD4_{183}$ were completely inactivated with respect to binding of both mAb 19Thy5D7 and Leu3a, indicating that CD4 is sensitive to adverse iodination or side reactions. The possible mechanisms of inactivation can be analyzed in terms of the known structure of $rsCD4_{183}$. $rsCD4_{183}$ is unlikely to be susceptible to the known oxidative side reactions of iodination (Koshland et al., 1963), as the only methionine is freely mobile at the N-terminus and all three tryptophans are buried. Histidine residues are known to be susceptible to iodination (Wolff and Covelli, 1969; Tsomides and Eisen, 1993; Sabatier et al., 1993) and $rsCD4_{183}$ has two histidine residues which are both exposed: H27 in D1 close to the gp120 binding site, and H107 in D2 distal to the gp120 binding site (Wang et al., 1990). Mutation of H27 to a smaller alanine residue is known to reduce binding of mAb 19Thy5D7 (Arthos et al., 1989; Moebius et al., 1992b) and the effect of adding a bulky iodine atom may be more extensive. The most likely mechanisms for inactivation at a high iodine:rsCD4 ratio are therefore that iodination of H27 might interfere sterically with binding of mAbs and gp120, or might change the conformation of other residues important for binding. Further studies would be needed to confirm this hypothesis.

Under trace-labeling conditions, where rsCD4 is in 10-fold molar excess over iodide, the predominant effects of iodination may be different and the analysis is more complex. The competition ELISA data show that under these conditions, the unlabeled WT $rsCD4_{183}$ molecules remained as active as F179Y, whereas the labeled WT molecules, assayed by immunoprecipitation, were much less active. Since inactivation by oxidative side reactions should affect labeled and unlabeled molecules similarly, the data confirm that oxidative side reactions cannot be responsible for the low activity of trace-labeled WT [^{125}I]-$rsCD4_{183}$. The immunoprecipitation data show that [^{125}I]-labeled WT $rsCD4_{183}$ molecules comprise an active and an inactive fraction, but that the inactive fraction is much greater than for F179Y. Based on the structural information, there are two possible mechanisms that could generate a mixture of active and inactive [^{125}I]-labeled WT $rsCD4_{183}$ molecules: (1) there may be a pre-existing small percentage ($\leq 10\%$) of non-native, inactive material which can be labeled at Y82; (2) native molecules can be iodinated on a residue other than Y82, such as H27

and H107. These reactions can also occur in F179Y $rsCD4_{183}$. However, under trace-labeling conditions where iodine is limiting, Y179 presumably competes intramolecularly for active iodine species that in WT $rsCD4_{183}$ engage in these other reactions. Consistent with this view, labeling of WT $rsCD4_{183}$ was much more strongly reduced by competition with free γ-Glu–Tyr than labeling of F179Y (Table 2), showing that the residue being iodinated in WT $rsCD4_{183}$ is less reactive than Y179 or present in only small amounts. As Y179 is accessible on native molecules and is distant from the binding site, effective competition by Y179 for active iodine species results in [^{125}I]-labeled F179Y $rsCD4_{183}$ preparations that are much more active than [^{125}I]-labeled WT $rsCD4_{183}$.

The device of introducing tyrosine residues into a protein has also been successfully applied to bovine interferon-τ (Li and Roberts, 1994). Trace-labeled protein (0.07 mol I/mol) was completely inactive in anti-viral assays, showing that this protein, in contrast to CD4, is highly susceptible to inactivation by oxidative side-reactions. Incorporation of two tyrosines in the C-terminal peptide region generated a protein that was more readily iodinated and retained activity upon iodination. Combining the results of that study with our results on rsCD4, we conclude that several distinct mechanisms may be responsible for apparent loss of activity upon iodination. The introduction of tyrosine residues at the C-terminus can improve the iodination properties of a protein both by introducing an iodinatable residue distant from the active site and by preventing the accumulation of active iodine species that may engage in undesirable iodinations or oxidative side reactions.

The F179Y $rsCD4_{183}$ construct clearly has improved iodination properties relative to either $rsCD4_{183}$ or $rsCD4_{371}$ and may prove useful in a number of applications requiring radiolabeled CD4. For instance, recent studies have shown that CD4-gp120 complexes bind with nanomolar affinity to chemokine receptors (Lapham et al., 1996; Trkola et al., 1996; Wu et al., 1996) and that soluble rCD4 can enhance the affinity of rgp120 for these receptors (Wu et al., 1996). Use of labeled CD4 in these studies would obviate the need for chemical labeling of gp120 with its potential effects on CD4-induced conformational changes in gp120. Other potential applications include CD4-gp120 binding studies, gel shift assays for inhibitors of CD4-gp120 binding, the selection of CD4-binding phage from phage display libraries in a plaque lift assay (Tsui et al., 1992) and on-bead screening of chemical diversity libraries (Wu et al., 1994) for CD4-binding compounds.

Acknowledgements

We thank Dr. J.H. Wang for crystallization tests, Dr. D. Littman for the HXB2 gp120 clone, S. Fountain and S. Iacono at Micromass for mass spectrometry analysis, Dr. R. Chopra and Dr. M. McGregor for help with analyzing solvent accessibility of tyrosines, and Dr. E.L. Reinherz, Dr. S.B. Easterbrook-Smith, Dr. A. Lew, Dr. C.I. Newbold, Dr. M.A. Recny, and Dr. A.T. Profy for reading the manuscript. We also thank many colleagues at Procept for invaluable help and support. This work was supported by National Institutes of Health Grant AI27336 (to E.L. Reinherz, in collaboration with S.C. Harrison and Procept) and by a postdoctoral fellowship from the CIRIT-Generalitat de Catalunya, Spain (to M.F.). S.C.H. is an investigator in the Howard Hughes Medical Institute.

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