Evidence for an Extended Structure of the T-cell Co-receptor CD8α as Deduced from the Hydrodynamic Properties of Soluble Forms of the Extracellular Region*

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We expressed soluble forms of the human T-cell coreceptor CD8α extracellular region, CD8α161, and the amino-terminal immunoglobulin-like domain, CD8α114, in Chinese hamster ovary cells and Escherichia coli, respectively. Both molecules were readily purified to homogeneity in milligram amounts and were recognized by a large panel of monoclonal antibodies. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis indicated that approximately 70% of CD8α161 was secreted as a disulfide-linked dimer, but CD8α114 was not disulfide-linked. To investigate the structural features of CD8α161 and CD8α114 under native conditions, we performed gel filtration and sucrose gradient sedimentation analysis. In spite of being partially or totally noncovalently bound, both recombinant molecules were stably associated homodimers, as no monomers could be detected at a fairly low protein concentration (~1 μM). This suggests that the CD8α amino-terminal domain alone strongly contributes to chain association. Determination of the Stokes radius (Rθ) and sedimentation coefficient (sθo,α) gave results consistent with CD8α114 having a globular shape and CD8α161 being an asymmetric molecule. Taking into account the contribution of hydration to the frictional coefficient, we obtained for CD8α161 an axial ratio of approximately 5, when modeled as a prolate ellipsoid. These results indicate that the elongated structure of CD8α161 is essentially contributed by the hinge region and help to explain how the CD8α is able to bridge the distance between the T-cell surface and its binding site in the αδ domain of major histocompatibility complex class I molecules on the target cell.

CD8 is a cell surface transmembrane glycoprotein expressed on a subset of T-cells that recognize peptide antigen presented by major histocompatibility complex class I molecules (1, 2). Antibody-blocking studies on cytotoxic T-cells (3) and gene transfer experiments in T-cell hybridomas (4, 5) have shown that CD8 is required for efficient antigen recognition and cellular activation. Several lines of evidence indicate that CD8 binds to class I molecules (6–8) and, in particular, to a nonpolymorphic site in the membrane-proximal αδ domain (9). Mutations in a 7-residue loop exposed to solvent in this domain have been found to affect CD8 binding (10, 11). Furthermore, studies with these mutants have shown that the simultaneous binding of CD8 and T-cell receptor to the same class I molecule on the target cell is essential for T-cell recognition and activation (10, 11), indicating that CD8 acts as a co-receptor. These data suggest that a ternary complex is formed in which the T-cell receptor interacts with the peptide antigen/αδ domains of class I, while CD8 is engaged in the recognition of a more distal site in the αδ domain.

CD8 is found on the cell surface as a disulfide-linked α/α homodimer and as a disulfide-linked α/β heterodimer (2). The CD8α chain (34–37 kDa) (12–14) is composed of an extracellular region containing an amino-terminal Ig-like domain of approximately 114 amino acids and a stretch of ~48 residues (hinge region) particularly rich in proline, threonine, and serine and heavily glycosylated by O-linked oligosaccharides (15, 16). This hinge region is followed by a hydrophobic transmembrane anchor and a short (28 residues) cytoplasmic tail. The latter contains a site of attachment for the src-related tyrosine kinase, p56lck (17, 18), thought to regulate signal transduction following engagement of CD8 with class I (19). X-ray crystallographic analysis of the CD8α amino-terminal domain indicates that it folds similar to an Ig variable domain and forms a homodimer (16). Mutation analysis of CD8α has provided initial evidence that this domain contains a binding site for class I (20). The CD8β chain (30 kDa) (21–23) which is expressed on the cell surface only if paired to the α chain, shows a similar overall structural organization, with the Ig-like domain containing, in addition, a J-like sequence (24). The CD8β chain does not appear to associate with p56lck (25).

The majority of class I-restricted T-cells express mostly the α/β heterodimer (21, 22) while α/α homodimers alone have been found on some gut intraepithelial T-cells (26), on some T-cell receptor γ/δ T-cells (27) and on natural killer cells (28). Several experiments have shown that the CD8α homodimer is sufficient to ensure binding to class I (6–8) and signal transduction (4, 5, 19), although recent evidence suggests that the CD8 α/β heterodimer confers a more efficient antigen-specific response (29).

In major histocompatibility complex class II-restricted T-cells the CD4 molecule is the functional homologue of CD8 (2). CD4 binds to class II molecules (30), and recent work has shown that CD4 interacts with the βδ domain of class II (31, 32) at a membrane-proximal site bearing structural homology...
to the CD8 binding site of class I. In contrast to CD8, however, CD4 is a monomer, and its extracellular region shows a different structural organization since it is formed of an array of four Ig-like domains. X-ray crystallographic studies of the first two Ig-like domains (33, 34), which bear the binding site for class II (35, 36), and additional biochemical and physicochemical studies (37, 38) strongly suggest that the extracellular region is an extended structure. This is likely to yield a molecule roughly equal in length to the sum of the T-cell receptor and part of class II (the α1 and β1 domains) interacting at the cell surface. Thus, the length of CD4 would be compatible with the first two Ig-like domains reaching the site of interaction with the β2 domain of class II.

How then is CD8, whose unique Ig-like domain is connected to the transmembrane segment through ~48 residues only, able to bridge a distance similar to the CD4 which contains four Ig-like domains? Although it has been proposed that the hinge region assumes an extended conformation (16, 39), and some support to this hypothesis comes from the observation that this region appears unstructured in the x-ray crystallographic analysis (16), no direct evidence has been provided to date. Thus, to investigate the structural basis for CD8 function, we expressed two secreted soluble forms of the CD8α chain, one comprising its entire extracellular region and the other, the Ig-like domain only, using CHO cells and Escherichia coli, respectively. We examined the hydrodynamic properties of these soluble forms of CD8α by gel filtration and sedimentation analyses. We show that both recombinant molecules are produced as stable homodimers, suggesting that the interaction between CD8α Ig-like domains provides an important contribution to chain association. Moreover, our data strongly suggest that the extracellular portion of the CD8α homodimer is an elongated structure, and this feature is essentially conferred by the hinge region. The successful expression of large quantities of the CD8 Ig-like domain should facilitate future structural studies.

**Materials and Methods**

**Monoclonal Antibodies**—The following anti-human CD8 mAbs were used: 21Ty-D23 (IgG1), (40), a gift of E. L. Reinherz (Dana Farber Cancer Institute, Boston); 4D12.1 (IgGl), 8.E1.7 (IgGZb), and 5'-GACTTCGCCAGTGATTAATACATCTGG-3'

Antibodies were used either purified or as ascities fluid.

**D. Olive (Institut Paoli-Calmette, Marseille, France);** B9.1 (IgG3), B9.2 (IgG3), B9.3 (IgG1), B9.4 (IgG1), B9.7 (IgG1), and B9.8 (IgG2.J, respectively.

The antibody mAb 48.9 (anti-β-galactosidase) was a gift from G. Winter (MRC, Cambridge, United Kingdom) cloned in M13mp19 was amplified using as a primer the reverse primer of M13, (M13RP) 5'-CAGGAAACAGCTATGAC-3'

**New England Biolabs), and the 3' primer oligonucleotide (primer 2), 5'-GACACCGGAATCTGCCATCTGGGTGGCCGG-3'.** The latter allowing amplification of the peptide lyase B sequence fused to the first six codons of the mature CD8α protein. A parallel amplification was carried out on CD8α161 DNA using the 5' primer oligonucleotide 5'-GCCCAACCAAGATGCGCCAGTCCGGGTGGCCGG-3' and the 3' primer oligonucleotide (primer 3) 5'-TGCGATGATCTGGGCTACGCTGGCAGGAGACCGG-3' (primer 4) hybridizing to the sequence corresponding to amino acids 108–114 of CD8α followed by a stop codon and a BglII site. The two amplified fragments were purified on agarose gel, mixed together, and subjected to a third PCR using the M13RF oligonucleotide and primer 4 for generating the chimeric construct peptide lyase B-CD8α114. The amplified DNA fragment was digested with HindIII and BglII, inserted into the vector pUC19, under the control of the inducible lacZ promoter, and introduced into the BMH 71–18 strain of E. coli. Each construct of CD8α was sequenced in its entirety by the dideoxy method (47).

**Transfection and Amplification in CHO Cells—Dihydrofolate reductase-negative CHO cell line DG44 (48), a gift of L. Chasin (Columbia University, New York), was grown in Ham’s F-12 medium (GIBCO) supplemented with 10% fetal calf serum; FCS, fetal calf serum; Fv, variable fragment; CHO, Chinese hamster ovary; mAb, monoclonal antibody; FCS, fetal calf serum; Fv, variable fragment; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; MTX, methotrexate.

The abbreviations used are: CHO, Chinese hamster ovary; mAb, monoclonal antibody; FCS, fetal calf serum; Fv, variable fragment; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; MTX, methotrexate.

**CD8α161 and CD8α114 Constructs—To obtain CD8α161, a 1-kilobase BamHI-FspI cDNA fragment encoding the human CD8α chain (13) was subcloned in M13mp19 and subjected to two sequential rounds of site-directed mutagenesis as described (45). Oligonucleotides 5'-TCCCTGGGGAGGGATCCATGGCCTTA-3' and 5'-GACCTGCCAGTATTAATACATCTGG-3' introduced a BamHI site immediately prior to the translation initiation codon and changed the codon for Ile-162 (ATC) to a stop codon, respectively. The latter oligonucleotide also changed the codon for Cys-160 (TGT) to a Ser codon (AGT). Mutated cDNA was digested with BamHI and XhoI (a unique site present in the 3'-untranslated region), made blunt by treatment with Klenow DNA polymerase and cloned into...
bacteria, an overnight culture at 37°C in 5 ml of 2YT medium containing 1% glucose and 200 μg/ml ampicillin was washed, resuspended in 5 ml of the same medium (without glucose) containing 1 mM isopropyl-1-thio-β-D-galactopyranoside, 100 μg/ml ampicillin, and 60 μCi/ml L-[^35]S]cysteine and incubated for an additional 3-4 h at 37°C. Periplasmic proteins were extracted as described (52), immunoprecipitated with anti-CD8 mAbs, and analyzed on a 14% acrylamide SDS gel. A panel of anti-CD8 mAbs was used to immunoprecipitate purified recombinant CD8α-soluble forms as above and the CD8α left in the supernatant was measured using the T8 cell-free enzyme-linked immunosorbent assay kit (T-cell Sciences).

Expression of CHO Cells and Purification of CD8α161—Roller bottles of 850 cm² were seeded with 2-3 × 10⁵ CHO cells expressing CD8α161 in 100 ml of complete Alpha medium with 4% FCS containing 50 mM of MTX and cells grown to confluence. This culture was maintained for 20 days by replacing the medium every 2 days. Cell supernatants were supplied with a mixture of protease inhibitors (see above), pooled, concentrated 5-fold on a tangential ultrafiltration Amicon system, and applied to a 20-ml Affi-Gel 10 column (Bio-Rad) coupled with 80 mg of the anti-CD8 mAb 01D11 coupled to Affi-Gel 10 beads. Washing and elution conditions were identical to those used in water 20% sucrose gradient in 100 mM NaCl, 20 mM Tris-HCl buffer, pH 7.5. Samples of 0.15 ml (at 0.5-6 mg/ml) were centrifugated in parallel with protein markers in an SW 41 rotor (Beckman) at 200,000 × g, for 29 h at 4°C. After centrifugation, the gradients were pumped through a Uvicord II (LKB) detector and proteins monitored at 280 nm. Fractions of 0.5 ml were collected and the percentage of sucrose measured with a refractometer (Zeiss). Protein peaks were analyzed by SDS-PAGE. The position of CD8α161 was determined by interpolation in a linear standard curve established with the following protein markers of known s₀₂₀ × W (54, 56): catalase (11.15 S), aldolase (7.6 S), chymotrypsinogen α (2.5 S), and ribonuclease A (2 S). To determine the partial specific volume (p) of CD8α161 we followed the method described by Muenzer et al. (57) by centrifuging CD8α161 as above onto two 5-20% sucrose gradients prepared in H₂O and in D₂O (99.5%, pure, a gift of Prof. M. Goldberg, Pasteur Institute).

Calculations of Partial Specific Volume, Molecular Mass, and p/p₀ Ratio—The partial specific volume (p) was calculated by using the following equation (57): p = ρ₀/(ρ₀ - ρ) = Kₗ₁/(Kₗ₂ - ρ), where K₁ and K₂ are the average densities of the sedimentation and diffusion coefficients in H₂O and D₂O (measured using a pycnometer at ρ/2 value for each protein). The molecular mass (m) of CD8α161 was deduced from the equation derived from Svedberg's and Stokes law: m = 6πηN₀k₀₁/1 - 1/ρ where η = 1 mPa.s is the viscosity and ρ = 1 g/ml is the density of water, at 20°C (58), and N₀ is Avogadro's number. The frictional ratio f/f₀ was calculated from the equation f/(f₀, D) = 0.354NA²(58).

RESULTS

Expression in CHO Cells of the Extracellular Region of CD8α in a Soluble Form—To express a soluble form of the human CD8α chain consisting of the extracellular region, CD8α161 (Fig. 1), the corresponding cDNA was subjected to site-directed mutagenesis which introduced a termination codon at Ile-162, predicted to be the first amino acid of the transmembrane region (13). Cells expressing CD8α161 were also used for the expression of a soluble form of the human CD8α chain (CD8αsol) as above. The position of the cysteine residues (c) is shown. CD8α161 was expressed in CHO cells and purified recombinant CD8α-soluble forms as above and the supernatant (periplasmic extract) was recovered, and protease inhibitors added and passed through an immunofraction column of 20 ml, containing 80 μg of the anti-CD8 mAb 10D11 coupled to Affi-Gel 10 beads. After washing and elution conditions were identical to those used for CD8α161. Eluted fractions containing CD8α161 were pooled and dialyzed against 20 mM sodium acetate buffer, pH 5.5, and applied to a CM-cellulose Memsep Cartridges (Millipore). After extensive washing with the same buffer, CD8α161 was eluted with 30 mM NaCl, 20 mM sodium acetate buffer, pH 5.5. The pH of the fractions was adjusted to 7.0 with 1 M Tris-HCl, pH 8, pooled, and concentrated using a Centricon 3 unit.

Amino Acid Sequencing—Purified CD8α161 and CD8α114 were electrophoresed under nonreducing conditions in a 10 and 14% acrylamide gel, respectively, and blotted onto Polybrene-coated glass fiber sheets (53). Amino acid amino-terminal sequence determination was carried out in an Applied Biosystem 470 gas-phase sequenator.

Determination of Soluble CD8α Stokes Radius—Samples of 0.1 ml containing purified CD8α161 and CD8α114 at the indicated concentrations were injected onto a Superose 6 HR 10/30 column (Pharmacia) connected to a fast protein liquid chromatography apparatus (Pharmacia) equilibrated with 100 mM NaCl, 20 mM Tris-HCl buffer, pH 7.5. Proteins were monitored at 280 nm and fractions analyzed by SDS-PAGE. Tryptic digestion was used as an internal control in each run. Stokes radii (Rₛ) of CD8α114 and CD8α161 were obtained using a linear relationship between Vₛ/Vₜ (Vₛ, total volume, Vₜ, elution volume) and Rₛ of the following proteins: catalase (6.22 nm), aldolase (4.81 nm), trypsinogen β (4 nm), horseradish peroxidase (3.02 nm), chymotrypsinogen α (2.09 nm), and ribonuclease A (1.84 nm). Purified Fv D1.3 (55), used as a calibrating standard at 2 mg/ml, was a gift of G. Boulot (Pasteur Institute).

Sedimentation Analysis—The sedimentation coefficients (sₛ) in water (sₓₛ) of CD8α161 was determined by using a preformed 5-20% sucrose gradient in 100 mM NaCl, 20 mM Tris-HCl buffer, pH 7.5. Samples of 0.15 ml (at 0.5-6 mg/ml) were centrifugated in parallel with protein markers in an SW 41 rotor (Beckman) at 200,000 × g, for 29 h at 4°C. After centrifugation, the gradients were pumped through a Uvicord II (LKB) detector and proteins monitored at 280 nm. Fractions of 0.5 ml were collected and the percentage of sucrose measured with a refractometer (Zeiss). Protein peaks were analyzed by SDS-PAGE. The position of CD8α161 was determined by interpolation in a linear standard curve established with the following protein markers of known sₓₛ (54, 56): catalase (11.15 S), aldolase (7.6 S), chymotrypsinogen α (2.5 S), and ribonuclease A (2 S). To determine the partial specific volume (p) of CD8α161 we followed the method described by Muenzer et al. (57) by centrifuging CD8α161 as above onto two 5-20% sucrose gradients prepared in H₂O and in D₂O (99.5%, pure, a gift of Prof. M. Goldberg, Pasteur Institute).

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Fig. 1. Schematic representation of truncated soluble forms of CD8α expressed in CHO cells and E. coli. The CD8α chain (mCD8α) is divided into boxes representing the signal peptide (L), the immunoglobulin-like domain (Ig-like), the hinge region (H), the transmembrane region (TM), and the cytoplasmic tail (Cyto.). Positive numbers indicate the amino acid residues corresponding to the approximate boundaries of each segment, according to Littman et al. (13). Negative numbers designate the beginning of the signal peptides. The position of the cysteine residues (c) is shown. CD8α161 was obtained by changing, by oligonucleotide-directed mutagenesis, the codons of Cys-160 to a stop codon and Ile-162 to a Ser (s) codon. CD8α114 was constructed by inserting a stop codon at Lys-115 and by replacing the sequence corresponding to the CD8α signal peptide with that of the peptidyl lysine signal peptide (pelB L) by PCR amplification using the procedure described under "Materials and Methods."
tated to Ser to avoid potential formation of illegitimate intrachain bonds with Cys-142 and to reduce the possibility of obtaining covalently linked multimers which have been observed for membrane-bound CD8 (15).

The modified CD8α cDNA was then subcloned into the pTG1566 dicistronic expression vector upstream of the dihydrofolate reductase gene. Expression of both CD8α161 and dihydrofolate reductase is driven by the adenovirus major late promoter to give a dicistronic mRNA. This results in a lower translation efficiency of dihydrofolate reductase compared with the upstream mRNA (59) and should allow a more efficient selection of clones expressing higher levels of CD8α161 at early stages of the gene amplification process.

This construct was transfected into dihydrofolate reductase-negative DG44 cells by the calcium phosphate method and clones selected initially in medium deprived of nucleosides and deoxynucleosides. A number of growing clones (21 clones) were screened for secreted CD8α protein by immunoprecipitation with anti-CD8 mAbs from the supernatant of L-[35S] cysteine-labeled transfectants and SDS-PAGE analysis. Secreted CD8α was detected as a 25-kDa species under reducing conditions (data not shown), the expected size for CD8α161. One clone secreting the highest levels of CD8α161 was subjected to amplification at progressively higher concentrations of MTX. Several rounds of selection (up to 50 nM MTX) allowed an overall improvement of approximately 70-fold in the level of secreted CD8α161 compared with unamplified cell line as assessed by quantitative immunoprecipitation and densitometric scanning of gel autoradiography (data not shown).

Biochemical Characterization of Purified CD8α161—Supernatants of the cell population selected at 50 nM MTX were concentrated by ultrafiltration, and CD8α161 was purified by affinity chromatography on an OKT8 mAb column followed by gel filtration on Sephadex G-150 or Superose 6 columns. The gel filtration step was necessary to remove a small fraction (about 10% of the affinity purified material) of aggregated CD8α161 and minor impurities which were eluted with the void volume. Typically, we obtained 2 mg of purified CD8α161 per liter of cell supernatants.

Analysis by SDS-PAGE, under nonreducing conditions of purified CD8α161 (Fig. 2A) shows a major species (approximately 70% of the material stained with Coomassie Blue) of an apparent molecular mass of 47 to 50 kDa and a minor one of 25 kDa. When reduced, the upper band migrates as a 25-kDa polypeptide chain. This indicates that a minor fraction of the secreted CD8α is not disulfide-linked. It is unlikely that the presence of the latter species is the result of proteolytic cleavage of part of the hinge region, including Cys-142, since its apparent molecular mass under nonreducing conditions is similar to that of the reduced form. The broad appearance of CD8α161 under reducing and nonreducing conditions is most likely because of the presence of differently glycosylated molecules. Amino acid sequence analysis of the 50–47-kDa and 25-kDa species, purified from nonreducing gels, detected the amino-terminal sequence (SQPVVR) predicted from the CD8α cDNA (13) (data not shown).

Gel filtration on Superose 6 shows that CD8α161 is eluted as a single homogeneous peak (Fig. 3A), indicating that no apparent dissociation of noncovalently bound molecule takes place under the conditions used. This was also true at dilutions of CD8α161 up to ~20 μg/ml (≈0.5 μM) (data not shown), indicating that non-disulfide-linked CD8α161 molecules are stably associated.

Table I shows that CD8α161 secreted by CHO cells is immunoprecipitated by 11 anti-CD8 mAbs, which recognize at least five distinct epitopes, suggesting no apparent alteration of the native conformation in the recombinant molecule. This is further reinforced by the fact that most of these mAbs did not detect CD8α161 in Western blot (data not shown).

The Hydrodynamic Properties of CD8α161 Indicate an Overall Elongated Shape—We determined the Stokes radius (Rs) of CD8α161 by gel filtration on a Superose 6 column, using a standard curve established with proteins of known Rs. CD8α161 was found to have a Rs of 4.0 ± 0.1 nm (Fig. 3B), which is close to that of the marker tryptophan synthase β2, a globular protein dimer of 88 kDa (60), almost twice the molecular mass of CD8α161 homodimers estimated by SDS-PAGE. One possible explanation for this result is that the overall shape of the extracellular domain of CD8α homodimer is rather asymmetric.

To provide support for this hypothesis, we carried out centrifugation analysis in isokinetic sucrose gradients to determine the sedimentation coefficient (s20, w) of CD8α161. As shown in Fig. 4A, a standard curve constructed with protein markers of known s20, w gave 2.5 ± 0.15 S for CD8α161. This is a rather low value for a globular protein of 50–47 kDa. Indeed, CD8α161 sedimented similarly to chymotrypsinogen α (25 kDa). Thus, together, gel filtration and sedimentation analyses are strongly suggestive of a molecule of pronounced asymmetric shape.

We next determined the partial specific volume (ρ) of CD8α161 according to the method described by Meunier et al. (57), which takes advantage of the differential migration of a macromolecule from the meniscus of sucrose gradients established in H2O and D2O (see "Materials and Methods" for details). By using this method (Fig. 4B) we determined for CD8α161 a ρ of 0.78 ± 0.01 cm3/g.

The experimentally determined Rs, s20, w, and ρ values were used in the equation derived by combining the Stokes law and

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**FIG. 2. SDS-PAGE analysis of purified CD8α161 and CD8α114. Panel A, CD8α161 was purified by affinity chromatography followed by gel filtration and analyzed on a 12% acrylamide gel. Approximately 10 μg of protein was loaded in each lane after boiling in sample buffer under nonreducing (NR) or reducing (R) conditions (20 mM dithiothreitol). 50 mM final concentration of iodoacetamide was added, before boiling, to nonreduced samples. The gel was stained with Coomassie Blue. Panel B, analysis on a 13% acrylamide gel of CD8α114 after purification on immunoadfinity column and subsequent ion exchange chromatography. Samples (10 μg) were treated as in panel A. Molecular mass (m) markers for panels A and B were bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), chymotrypsinogen α (25 kDa), soybean trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), and ribonuclease A (13.6 kDa).**
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FIG. 3. Gel filtration chromatography and determination of the Stokes radius for purified CD8α161 and CD8α114. Gel filtration analysis was performed on a Superose 6 HR 10/30 column equilibrated with a 100 mM NaCl, 20 mM Tris-HCl buffer, pH 7.5, using a fast performance liquid chromatography system (Pharmacia). Elution profiles of purified CD8α161 (panel A) and CD8α114 (panel C) were recorded at 280 nm. Samples of 0.1 ml each, containing purified CD8α161 (~5 mg/ml) or CD8α114 (~1 mg/ml) or protein markers (~1 mg/ml) were loaded on the column and eluted at a flow rate of 0.5 ml/min at room temperature. Stokes radii (Rs) of CD8α161 (open triangle in panel B) and CD8α114 (open circle in panel D) were determined from standard curves obtained by using protein markers which were: peroxidase (0.02 nm), tryptophan synthase β (4 nm), aldolase (4.81 nm), and catalase (5.22 nm) for CD8α161 (closed squares in panel B, in the order of increasing Rs) and ribonuclease A (2.96 nm), peroxidase (3.02 nm) and catalase (5.22 nm) for CD8α114 (closed circles in panel D). Dimers: Fv D1.3, used at a concentration of 2 mg/ml, is indicated by an open square in panel D. V0 (exclusion volume) was 7.3 ml, Vt (total volume) was 22.4 ml, Vc (elution volume) for CD8α161 was 16.5 ml and was 18.7 ml for CD8α114. Vc determinations for CD8α161 and CD8α114 were performed four and three times, respectively. The standard deviation of the curve was ±0.1 nm for CD8α161 and ±0.2 nm for CD8α114.

TABLE I
Reactivity of CD8α161 and CD8α114 with a panel of anti-CD8α mAbs

| Anti-CD8α mAbs | CD8α161 | CD8α114 |
|----------------|---------|---------|
| 21Thy-2D3     | ++      | +       |
| B9.3          | ++      | +       |
| 10D11.5       | +       | +       |
| 4D12.1        | +       | +       |
| 8.E1.7        | +       | +       |
| 10B4.6        | +       | +       |
| OKT8          | +       | +       |
| B9.8          | +       | +       |
| B9.7          | +       | +       |
| B9.4          | +       | +       |
| B9.1          | +       | +       |

* +, immunoprecipitation ≥ 80–100% of CD8α161 and CD8α114.
* −, immunoprecipitation ≤ 5% of CD8α161 and CD8α114.

From the above data we could calculate a fractional ratio f f/ f0 of 1.6 for CD8α161 (see “Materials and Methods”), a value indicative of an extended structure.

Expression of the Ig-like Domain of CD8α, CD8α114, in E. coli: Purification, Gel Filtration, and Sedimentation Analyses—The amino-terminal domain of CD8α chain has been shown to fold as an Ig variable domain. It was therefore interesting to compare its hydrodynamic properties with those of the entire extracellular region of CD8α. This should allow us to understand which one of the CD8α161 regions is responsible for the observed hydrodynamic properties and to further validate the experiments reported above.

For the purpose of the present study and, at the same time, to obtain a recombinant molecule which could be advantageous for further structural studies, we attempted expression of the amino-terminal domain of CD8α in CHO cells. The CD8α CDNA was modified by introducing a termination codon at Lys-115 using PCR amplification. This should result in expression of only the predicted Ig-like domain of CD8α, CD8α114. However, expression in CHO cells using the pTG1563 vector did not give detectable secreted protein. Expression in E. coli was therefore tried by replacing the signal sequence present in CD8α114 with that of the bacterial protein pectate lyase (Fig. 1) as described under “Materials and Methods.” This strategy has been used previously for obtaining secretion of eukaryotic proteins, including Fv frag-

the Svedberg equation, to calculate a molecular mass of approximately 51 ± 4 kDa for CD8α161. This value is in close agreement with the estimation made on SDS-PAGE and the size of two CD8α161 polypeptide chains deduced from the primary structure plus their carbohydrate content (16). In addition, this result excludes that the elution volume observed for CD8α161 in gel filtration could be attributed to the recombinant molecule forming tetramers (e.g. two stably associated homodimers of CD8α161 making 90–100 kDa) composed of dimers having an overall globular shape.
molecules of Ig (55), in the periplasmic space of E. coli. The pectate lyase B-CD8α114 DNA construct was subcloned into pUC19 under the control of the lacZ-inducible promoter and used for transformation of E. coli. Following induction with isopropyl β-D-galactopyranoside, CD8α114 could be detected in E. coli periplasmic space after labeling with L-[35S]cysteine and immunoprecipitation with anti-CD8 mAbs (data not shown).

Large scale purification of CD8α114 by affinity chromatography on an anti-CD8 mAb (10D11.5) column followed by cation exchange chromatography gave 300–500 μg of purified protein/liter of bacteria. Monoclonal antibody 10D11.5 was used instead of OKT8 since the latter did not react with CD8α114 (see Table I).

The purified CD8α114 molecule migrates in SDS-PAGE as a 12-kDa species in both reducing and nonreducing conditions (Fig. 2B), in close agreement with the size of the polypeptide chain predicted from the DNA sequence. Amino-terminal amino acid sequencing indicated that the pectate lyase B signal sequence had been cleaved at the predicted amino terminus of CD8α (data not shown).

CD8α114 was recognized by 10 of 11 anti-CD8 mAbs (Table I), suggesting that its conformation is close to the native one and that the epitope recognized by the unreactive mAb OKT8 is within or very near to the hinge region.

Fig. 3C shows that CD8α114 was eluted from a Superose 6 column as a single homogeneous peak and had a Rs of 2.3 ± 0.2 nm (Fig. 3D). CD8α114 is eluted almost at the same position as chymotrypsinogen a (25 kDa) and as an Ig Fv (VH + VL) dimer (~25 kDa, Rs 2.2 ± 0.2 nm). When analyzed by sedimentation on a 5–20% sucrose gradient at low protein concentration (10–20 μg/ml), CD8α114 was detected as a single homogeneous peak with a sedimentation coefficient of 25 ± 0.15 S, almost identical to the value observed for the Ig Fv, 2.4 ± 0.15 S (data not shown), further confirming that they share similar hydrodynamic properties. By assuming a s of 0.73 cm²/g (a typical value for most unglycosylated proteins) (56) a molecular mass of 22 kDa can be deduced for CD8α114, fully compatible with it being an homodimer. Thus, similar to the Ig Fv, CD8α114 appears to be a stable dimer since no monomer could be detected at fairly low protein concentrations (20 μg/ml ≈ 1 μM). This observation suggests therefore that the Ig-like domain may be sufficient per se to ensure a strong interchain interaction in the dimer formed by the entire extracellular region.

In addition, the above data indicate that the CD8α hinge region confers unique hydrodynamic properties consistent with an elongated structure of the CD8α homodimer.

**DISCUSSION**

To provide further insight into the structure and function of the CD8α co-receptor, we investigated its hydrodynamic properties and chain association requirements. This was made possible by the successful expression of soluble forms of the CD8α chain extracellular region, CD8α161, in CHO cells and of the Ig-like domain, CD8α114, in E. coli. Both recombinant molecules were readily purified in milligram amounts. Analysis by SDSPAGE, gel filtration, and sedimentation on sucrose gradients indicated that they were both secreted as homodimers, the former being in large part (≥70%) disulfide-linked, whereas the latter, as expected (16), was not.

Several lines of evidence suggest that the structure of recombinant CD8α161 and CD8α114 is close to the native one. First, 10 mAbs tested by immunoprecipitation recognized the recombinant molecules. OKT8 mAb reacted only with CD8α161 and is presumably directed at an epitope in the hinge region. Second, the pattern of proteolysis of CD8α161 with different proteases closely resembled the pattern observed for the membrane-bound form (62). Third, the purified recombinant molecules consistently gave homogeneous peaks in both gel filtration and sedimentation analysis, and no spontaneous aggregates were detected.

The presence of a fraction of CD8α114 dimers non-disulfide-linked could not be attributed to proteolytic removal of part of the hinge (at least 20 residues) including Cys-142, during expression or purification. Non-disulfide-linked CD8α114 dimers might be caused by the absence of Cys-160 in our construct implying perhaps that membrane-bound CD8α may exist in different isoforms which use one or the other (or both) cysteines to form interchain disulfide bridges.

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7 R. A. Mariuzza, personal communication.
However, others have reported that a soluble extracellular region of human CD8a (CD8α162) expressed in CHO cells and containing both cysteines, paradoxically, did not form interchain disulfide bonds (16). A likely explanation for these observations is that, as we anticipated when designing our constructs, illegitimate intrachain disulfide bonds are formed which, surprisingly, may be favored in the soluble molecule.

Similar to our finding, it was found that a soluble human CD8α, truncated at residue 146 (including only one cysteine of the hinge), contained a fraction of non-disulfide-linked homodimers (16). Together, these observations suggest that the hinge region possesses a high degree of flexibility. The presence of the membrane anchor in the intact molecule may, however, facilitate chain proximity, thus favoring correct and quantitative formation of disulfide bonds.

Covalent bonding of CD8α161 dimers may not be essential to maintain association since monomers, which could be derived from the non-disulfide-linked molecules, were not detected by gel filtration and sedimentation analysis even at micromolar protein concentrations. Likewise, CD8α114 dimers, at similar protein concentrations, were found to be stably associated. Although the behavior in solution of CD8α114 is in agreement with the crystallographic model indicating that the two Ig-like domains of CD8α form an Fv-like dimer via multiple contacts at the interfaces (16), its stability in the absence of the hinge region, observed here, suggests that the latter may only marginally contribute to dimer association and/or stabilization.

CD8α161 was found to have a Rs of 4.0 ± 0.1 nm and a s20,w of 2.5 ± 0.15 S, both values highly inconsistent with a globular protein having the molecular mass of the dimer estimated in SDS gel. By using these parameters and the experimentally determined partial specific volume of CD8α161, the molecular mass was found to be 51 kDa, in close agreement with the size of two polypeptide chains of 17.5 kDa with 14 O-linked carbohydrates (16), allowing an average of 1 kDa/carbohydrate chain. Furthermore, the hinge region appears to largely contribute to the hydrodynamic properties of CD8α since CD8α114 shows a Rs and s20,w similar to those of an Ig Fv dimer, whose three-dimensional structure is known (61).

A frictional ratio f/fo of 1.6 was calculated for CD8α161. However, this value must be corrected for the hydration coefficient (δ), which may be high for a molecule of elongated structure and containing, at the same time, carbohydrate chains which tend to be highly hydrated. For CD8α161 the carbohydrate content was estimated to be approximately 30% of its total mass. Typical values for most proteins can vary between 0.1 and 0.6 g of H2O/g of protein, but more extreme values of 0.8 have been reported (56). Thus, when a δ between 0.6 and 0.8 was used, to take into account high carbohydrate content, we calculated an axial ratio of 6 to 5 for CD8α161, assuming a prolate ellipsoid (58). In this respect, a meaningful comparison can be made with the extracellular region of CD4 whose f/fo was also found to be 1.6, and the axial ratio was roughly 6, after correction for hydration (37). From these studies and from x-ray crystallographic data (33, 34, 37), the length of CD4 extracellular domain has been predicted to be ~14 nm.

We could calculate for CD8α161 a maximal theoretical length of 18–20 nm for a prolate ellipsoid model (58), sufficient for the Ig-like domain to reach the αo domain of class I. This implies that, after subtracting the length of the Ig-like domain (2.5–3 nm), the hinge region can be approximately 15–17 nm long. Although these values must be regarded as indicative because of the uncertainties about the molecular model (prolate ellipsoid) and the hydration coefficient asumed, the primary structure of the hinge region and additional considerations may be help to explain these results. The presence of 10 prolines scattered throughout this region can be expected to disrupt formation of α-helices and/or β-strands often and suggests that the corresponding polypeptide chain may be, partially, a random coil. Indeed, circular dichroism spectra of CD8α161 (data not shown) tend to support this hypothesis. Furthermore, the hinge region contains several O-linked sialylated carbohydrate chains, on threonine and serine residues, a feature found in a number of proteins of extended structure (63–66). At least in the case of mucins, which have been well studied, it was found that O-linked oligosaccharides were required for maintaining an extended conformation of the polypeptide chain (63), and it was predicted that the segments of the molecule bearing carbohydrate chains should have an average length of ~0.25 nm/residue. If part of the CD8α hinge region (48 residues) will have a length/residue between this value and the theoretical value of 0.36 nm/residue, for a fully extended polypeptide backbone conformation, it would be possible for the entire extracellular domain to have a longitudinal dimension close to the calculated one.

Finally, the absence of electron density for the first 27 residues of the hinge region present in the crystalized molecule (16) indicates that they possess a high degree of mobility. Thus, it can be speculated that although the Ig-like domain provides stable association of the dimer, as also suggested by our observations, loose or no interchain contacts within a large section of the hinge region may favor a high conformational freedom of this region. This may facilitate CD8 flexibility and therefore the capacity to reach the ligand on the target cell.

As also reported by others for human (16) and rat (39) CD8α, we were unable to detect secreted CD8α114 in CHO cells, suggesting that the hinge region plays a role in facilitating the intracellular transport of CD8α. However, CD8α114 expressed with the bacterial pectate lyase B leader sequence or with its authentic leader sequence was secreted in the periplasmic space of E. coli or in supernatants of SF9 insect cells using recombinant baculovirus, respectively. The reason for the different fate of CD8α114 in CHO cells versus E. coli and insect cells is unclear, but it may be related to the phylogenically distant origin of the host cells whose secretory machinery may exert more or less stringent controls (e.g., transport, retention, degradation) on foreign proteins. That this may, as a consequence, result in the production of differently folded CD8α in different cellular environments is not supported by the fact that truncated versions of CD8α expressed in different hosts were equally reactive with a large panel of specific mAbs.

The expression in E. coli of the CD8α114 dimer in large quantities has applications for structural studies. This recombinant molecule needs no further treatment for deglycosylation and protease digestion as has been the case for a truncated CD8α146 expressed in CHO cells utilized for x-ray crystallographic analysis (16) and may prove useful for future attempts to co-crystallize CD8 with class I. Furthermore, this approach may be applied to the expression of the amino- terminal domain of CD8 α/β dimer, the most abundant and perhaps more physiological form of CD8 expressed on T-cells. These recombinant molecules are currently under construction.

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1. Laisney, J. P. Boursier, F. Hervé, and O. Acuto, unpublished data.
