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Dependence of Conformation of D3/D4 Domains of Human CD4 on Glycosylation and Membrane Attachment

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Abstract—Conformational dynamics of human T-helper cell receptor protein CD4 has been studied with the help of monoclonal antibody (mAb) T6. The mAb T6 discriminates between s- and m-forms of CD4 and recognizes a specific conformation of the soluble (s) form of CD4 including the first nine amino acids of CD4 transmembrane sequence. However, change of tryptophan for serine in position 2 in this sequence destabilizes the T6-type conformation. By enzymatic deglycosylation and deletions of glycosylation sites, we show that T6-type conformation depends on glycosylation in both sites (Asn271 and Asn300). We show also that the sugars are not involved in direct binding to the antibody but stabilize the D3/D4 local conformation. Deglycosylated forms of sCD4 *in vivo* acquire a specific conformation similar to the wild type sCD4, which however cannot be restored after denaturation/renaturation under conditions of non-reducing Western blot. This observation indicates that the correct protein folding needs chaperone assistance and cannot be achieved *in vitro*. Completely non-glycosylated sCD4 is synthesized and secreted into the growth medium. In the medium, this mutant appears to be unstable and aggregates during time. In a contrast to soluble CD4, mutations in glycosylation sites abrogate expression of membrane CD4, thus demonstrating a different secretion pathways for soluble and membrane proteins.

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Key words: CD4, autoimmune epitope, glycosylation, protein folding, HIV-1

Receptor protein CD4 is known to be present on the surface of T-lymphocytes, cells of the monocyte-macrophage lineage, and some other cells such as dendritic antigen-presenting cells. CD4 binds MHC (major histocompatibility complex) class II molecules. CD4 is involved in signal transduction through binding its cytoplasmic domain to p56lck, a cytoplasmic tyrosine-kinase. CD4 and T-cell receptor binding to the same MHC-ligand [1] commences a synergetic co-receptor signaling process, which activates T-cells, and, in turn, leads to the activation of B-lymphocytes in the humoral immune response and CD8-cells in the cytotoxic immune response [2, 3].

Receptor protein CD4 is a single-chain type I integral membrane glycoprotein composed of four immunoglobulin-like domains (D1-D4) and containing six cysteine residues that form three intra-chain disulfide bonds. The extracellular region of the molecule consists of 370 amino acid residues (a.a.), followed by a transmembrane sequence and a short cytoplasmic domain (25 and 38 a.a., respectively) [4]. The extracellular domain contains two potential sites for N-glycosylation (Asn271 and Asn300, located between the D3 and D4 domains). Both sites have been shown to have oligosaccharides attached [5].

In addition to the common membrane form of CD4 (mCD4), a soluble form of receptor, barely detectable in sera of healthy individuals, has been reported in a variety of pathological states. Soluble CD4 (sCD4) has been reported to be secreted into the serum of patients infected with some viruses, such as human immunodeficiency virus (HIV-1) [3, 6] or Epstein–Barr virus [7]. Soluble CD4 is detected also in sera of patients suffering from other diseases, particularly autoimmune diseases [8-12].

The sCD4 molecule in a complex with HIV envelope protein can be an autoantigen inducing an antibody response to CD4 in human-CD4 transgenic mice. It was produced by mouse monoclonal antibody (mAb) T6, with

Abbreviations: a.a., amino acid residue; IL, interleukin; mAb, monoclonal antibody; MHC, major histocompatibility complex; PBS, phosphate buffered saline; sCD4 and mCD4, soluble and membrane forms of CD4, respectively.

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an epitope located in the D3/D4 domains of CD4 that discriminates between soluble and membrane-associated receptor forms [13, 14]. These antibodies were shown to compete with anti-CD4 autoimmune antibodies found in ~15% of HIV-1 patients [15-17]. In this article, using mAb T6, we demonstrate that the D3/D4 domain region of sCD4 has a specific local conformation stabilized by sugar moieties and recognized by autoimmune antibodies.

MATERIALS AND METHODS

Reagents. Recombinant human sCD4 was produced by SmithKline Beecham (Germany). Mouse anti-human CD4 mAbs (CG9, CG76, T6, and 7B4) were kindly provided by Dr. G. Denisova (Department of Cell Research and Immunology, Tel-Aviv University, Israel) [18]. FITC-conjugated anti-mouse IgG was purchased from PharMingen, Becton Dickinson Company (USA). Goat anti-mouse and goat anti-human IgG conjugated with alkaline phosphatase or horseradish peroxidase (HRP) were purchased from Jackson Immunoresearch Laboratories (USA), and N-glycosidase F from Roche Molecular Biochemicals (USA). Sera of HIV-1 positive patients were obtained from Dr. E. V. Kazennova (Ivanovsky Institute of Virology, Russian Academy of Medical Sciences, Moscow, Russia).

Plasmid expressing full-length CD4 (1-433 a.a.) was kindly provided by Dr. M. Federico [19]. Plasmid expressing "tailed" sCD4 (1-372 a.a.) was constructed by insertion into vector pcDNA3 of an EcoRI/SalI fragment encoding the D1-D4 domains of CD4. The fragment was obtained from plasmid pCDLTMI, kindly provided by Dr. C. Broder [20].

Plasmid expressing sCD4 (1-363 a.a.) was constructed by mutational insertion of a stop codon immediately after the Pro363 residue of mCD4 using primers st1 (5′AGGTTCTGCCC**TGA**TGGTCC3′) and st2 (5′GGACCA**TCA**GGGCAGAACCT3′). The schemes of the plasmid inserts are presented in Fig. 1, a and b.

The N-glycosylation sites of CD4 were eliminated via alanine substitution of Asn271 and/or Asn300 in sCD4 and mCD4 expressing vectors using primers: 271_1 (5′CTGGCTCTGGA**GCC**CTCACCCTG3′), 271_2 (5′CAGGGTGAG**GGC**TCCAGAGCCAG3′), 300_1 (5′CAGCTCCAGAAA**GCT**TTGACCTGTGAG3′), and 300_2 (5′CTCACAGGTCAA**AGC**TTTCTGGAGCTG3′). The mutagenesis procedure was based on the Stratagene Cloning Systems Quick Change kit (USA).

Double stranded DNAs were isolated using the QIAGEN QIAprep Spin Mini- and Maxiprep (Germany).

Transfection of 293T cells was performed by the standard calcium phosphate method.

The procedures of cell lysis, immunoprecipitation, Western blotting, and immunofluorescence analysis were performed as described in [14].

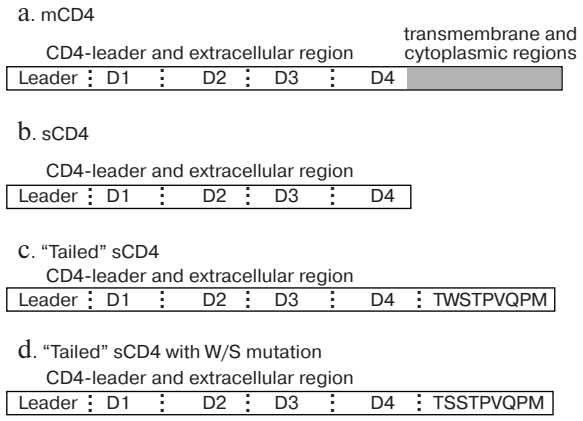

Fig. 1. Schemes of plasmid inserts.

Radio-active "pulse-chase" analysis of transfected cells. Twenty hours following the transfection, the cells were rinsed and incubated with Cys/Met free media plus dialyzed fetal calf serum (10%) for 30 min. Then the cells were incubated in the same medium containing a mixture of ^{35}S-labeled Cys/^{35}S-labeled Met (0.5 mCi/ml) for 40 min at 37°C. After the pulse, the cells were washed with phosphate buffered saline (PBS) and incubated with normal DMEM medium for the next 16 h. The collected media and/or the cell lysates were incubated with mAb CG9 or T6 attached to Sepharose beads. The immunoprecipitated proteins were separated in 12% SDS-polyacrylamide gel. The gel was fixed for 10 min in acetic acid, soaked for 30 min in fluorophore (20% (w/v) PPO (2,5-diphenyloxazole) in acetic acid), washed, dried, and exposed to X-ray film.

Coupling of ligands to CNBr-activated Sepharose 4B beads. Conjugates of Sepharose with mAbs T6 and CG9 were prepared according to a protocol recommended by Pharmacia Fine Chemical Co. (Sweden).

Deglycosylation. The N-glycosidase F treatment of sCD4 was conducted in 100 μl of 0.1 M phosphate buffer (pH 8.0) in the presence of 1 μl of Complete Protease Inhibitor Cocktail (Roche Molecular Biochemicals) overnight at 37°C.

RESULTS

Involvement of N-linked glycans in formation of T6 epitope within sCD4. In our previous paper [14] we demonstrated that treatment of sCD4 with N-glycosidase, which removes oligosaccharide chains from the protein, does not influence its recognition by CG9 mAb (the epitope is localized in the D1 domain) but completely destroys the epitope of T6 under the conditions of non-reducing Western blot. Similar results were obtained in studying the dependence of 10 HIV-infected patients'

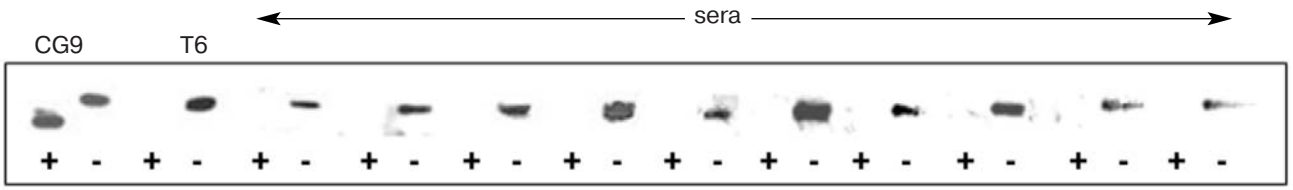

Fig. 2. Effect of sCD4 deglycosylation on sCD4 binding to autoimmune human sera. sCD4 (1 mg/ml in PBS) was incubated in the presence (+) or absence (–) of N-glycosidase F. Samples (0.15 μg sCD4 in each lane) were run in 12% SDS-PAGE, transferred onto nitrocellulose membranes, and incubated with mAbs CG9 and T6 and 10 different sera from HIV infected individuals with autoimmune CD4 response (diluted 1 : 1000). Membranes were incubated with anti-mouse or anti-human IgG HRP conjugate (1 : 2500).

CD4 autoimmune serum activity on sCD4 glycosylation (Fig. 2).

To study the influence of glycosylation under native condition of the protein, we designed two plasmids encoding partially-glycosylated proteins (Δ271 or Δ300) and one plasmid encoding non-glycosylated sCD4 (Δ271/300). The strategy was to prevent N-glycosylation by mutating each or both of the asparagine residues (271 and 300, which are part of the consensus sequence Asn-X-Thr/Ser and to which carbohydrates are covalently linked) to alanine residues. 293T cells were transfected with these plasmids. To follow the synthesis and secretion of sCD4 forms, we performed pulse/chase analysis 20 h following the transfection. The proteins from cells and media were immunoprecipitated with mAb CG9 attached to Sepharose beads and separated by 12% SDS-PAGE. The dried gel exposed X-ray film. The results are presented in Fig. 3. As can be seen, mutant forms of sCD4 were expressed by the cells with various efficiency. The absence of one glycosylation site decreases the expression of the corresponding mutant protein (~50%). In the absence of both glycosylation sites, the expression is even lower (<30%). However, all the forms of sCD4 were detected in the media 16 h after the pulse (Fig. 3).

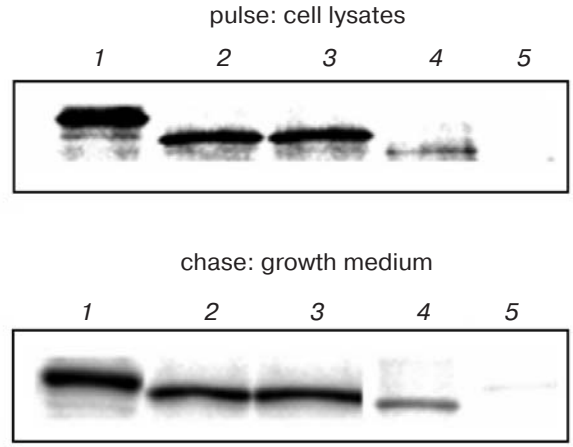

Fig. 3. Pulse-chase analysis of sCD4 synthesis and secretion. 293T cells were transfected the plasmids encoding glycosylated (*1*), partially-glycosylated Δ271 (*2*) and Δ300 (*3*), and non-glycosylated (*4*) sCD4 and vector plasmid k (*5*). After the pulse half of the cells were lysed, and the other half was chased in normal DMEM medium for 16 h and the media were collected for analysis. Then sCD4 from the cell lysates and media were immunoprecipitated with mAb CG9 attached to Sepharose beads. The precipitates were separated in 12% SDS-PAGE. After fixation the gel was soaked in fluorophore, dried, and exposed to X-ray film.

To test the recognition of the sCD4 glycosylation mutants by mAb T6 inside transfected cells, we performed immunofluorescence staining. The results are presented in Fig. 4. One can see that CG9 mAb detects all sCD4 forms, and T6 mAb fails to bind only the double mutant. The same is true for another anti-CD4 mAb, CG76, which has its epitope in D3 domain of CD4 and is also sugar-dependent (data not shown). These results demonstrate that the absence of sugar moieties in a single position does not significantly change the intracellular local conformation of sCD4. But in the absence of glycosylation in both sites the conformational epitopes of anti-D3 and anti-D4 domain mAbs are not exposed intracellularly. Probably they are masked by cellular chaperones, which stabilize the sCD4 structure in the absence of sugars. In contrast to sCD4, the membrane form of CD4 with deleted glycosylation sites was not detected in cells by staining with mAbs CG9, CG76, and T6 (data not shown).

To evaluate the synthesis and secretion of the sCD4 mutated forms, 48 h after the transfection the cells were lysed and the lysates were analyzed by Western blot. The growth media were also collected and analyzed by immunodot and Western blot assays. The results are summarized in Fig. 5.

To quantify the synthesized sCD4 two mAbs were used: CG9 that has the epitope within D1 domain, and 7B4 developed against denatured CD4 and recognizing epitope ELWWQAE located in D3 domain. The epitopes of these mAbs were shown previously not to be influenced by enzymatic deglycosylation (data not shown). As we see in Fig. 5a, the CG9 and 7B4 mAbs show the same ratio in various sCD4 forms in the cell lysates. However, under the same conditions T6 mAb recognizes only wild type sCD4 and does not react with the CD4 mutants. These results thus show that the epitope in partially or completely non-glycosylated sCD4 is not presented under the Western blot conditions.

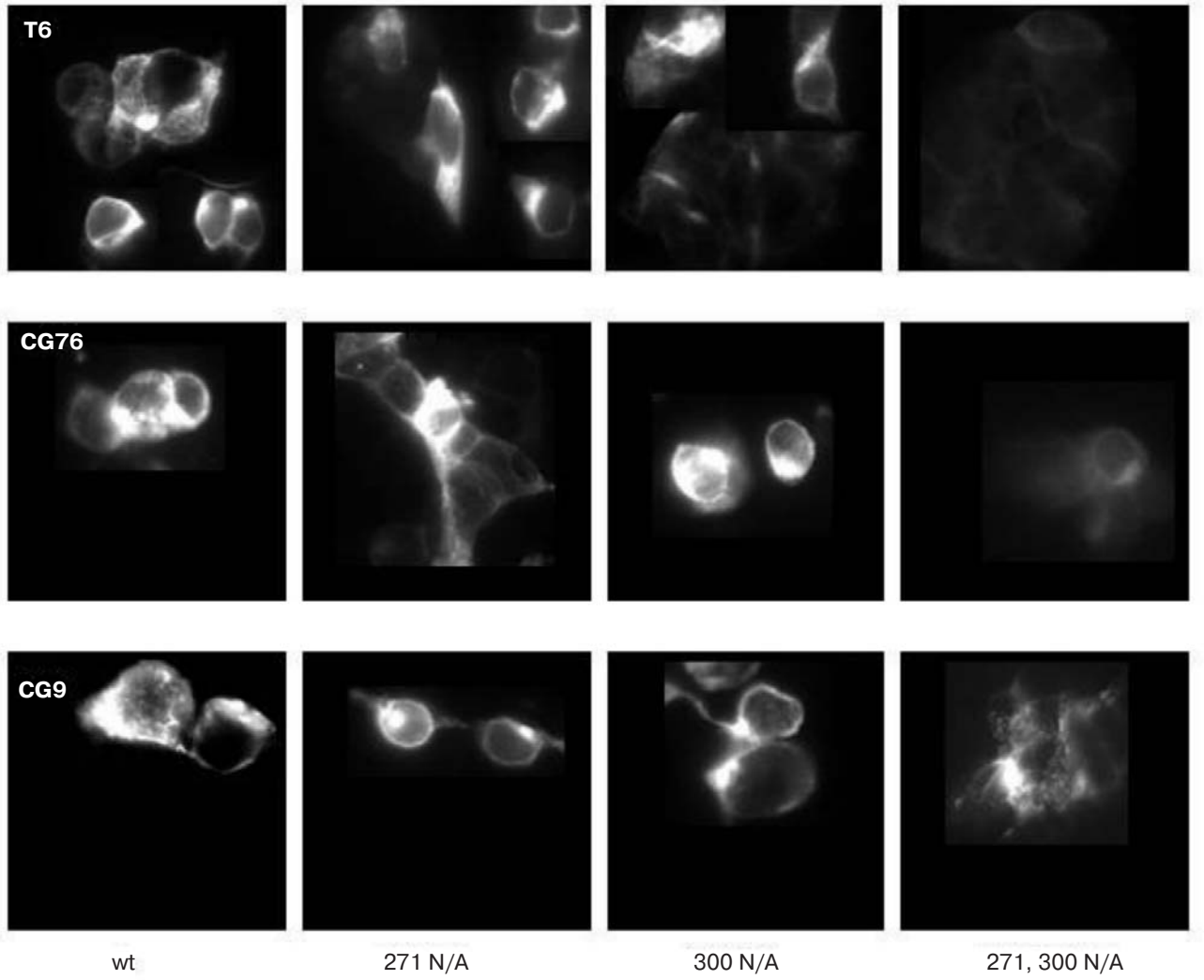

Fig. 4. Immunohistochemistry of non-glycosylated sCD4. 293T cells were transfected with plasmids encoding wild type (wt), mutant Δ271, Δ300, or Δ271/300 sCD4 on coverslips. In 48 h following the transfection, the cells were fixed with 3% *p*-formaldehyde, permeabilized with Triton X-100, incubated with mAbs CG9, CG76, or T6, and labeled with anti-mouse IgG mAb conjugated with FITC (1 : 500). Digital photography was performed using an imaging system on a Leica fluorescence microscope. The whole samples were analyzed and typical images are presented.

Analysis of sCD4 secreted into the media shows that the levels of secretion of the mutated forms are slightly lower than that of wild type (Fig. 5b). The sCD4 Δ271/300 mutant, which does not contain any sugar residues, accumulates in the media, preferentially in aggregated form (Fig. 5b). The mAb T6 does not recognize in Western blot the secreted mutant Δ271 and Δ300 sCD4. However, it recognizes the aggregated form of sCD4 Δ271/300 double mutant. Thus, the aggregation stabilizes the T6-type conformation of this mutant. When analyzed by dot blot (Fig. 5c) or immunoprecipitation (Fig. 5d), where the proteins are in native conditions, all the secreted sCD4 forms were recognized by T6 mAb but with lower efficiency compared with the wild type sCD4. This result proves our previous finding that glycosylation is necessary not for the epitope recognition but rather for stabilization of the native conformation. In the absence of sugar, the protein undergoes irreversible denaturation under the conditions of non-reducing Western blot analysis.

The aggregated double mutant CD4 could be detected only 24-48 h after transfection, and after 72 h it is already not detectable with any of the mAbs which we used: CG9, CG76, T6, and 7B4 (data not shown). Probably the process of aggregation continues in the media and upon the long incubation the aggregate becomes insoluble.

Influence of C-terminus extension on folding of soluble form of CD4. It has been shown previously that T6 mAb does not bind mCD4 either in cells or in Western blot analysis [14]. Therefore, we ask whether just the presence of the anchor sequence in the mCD4 molecule

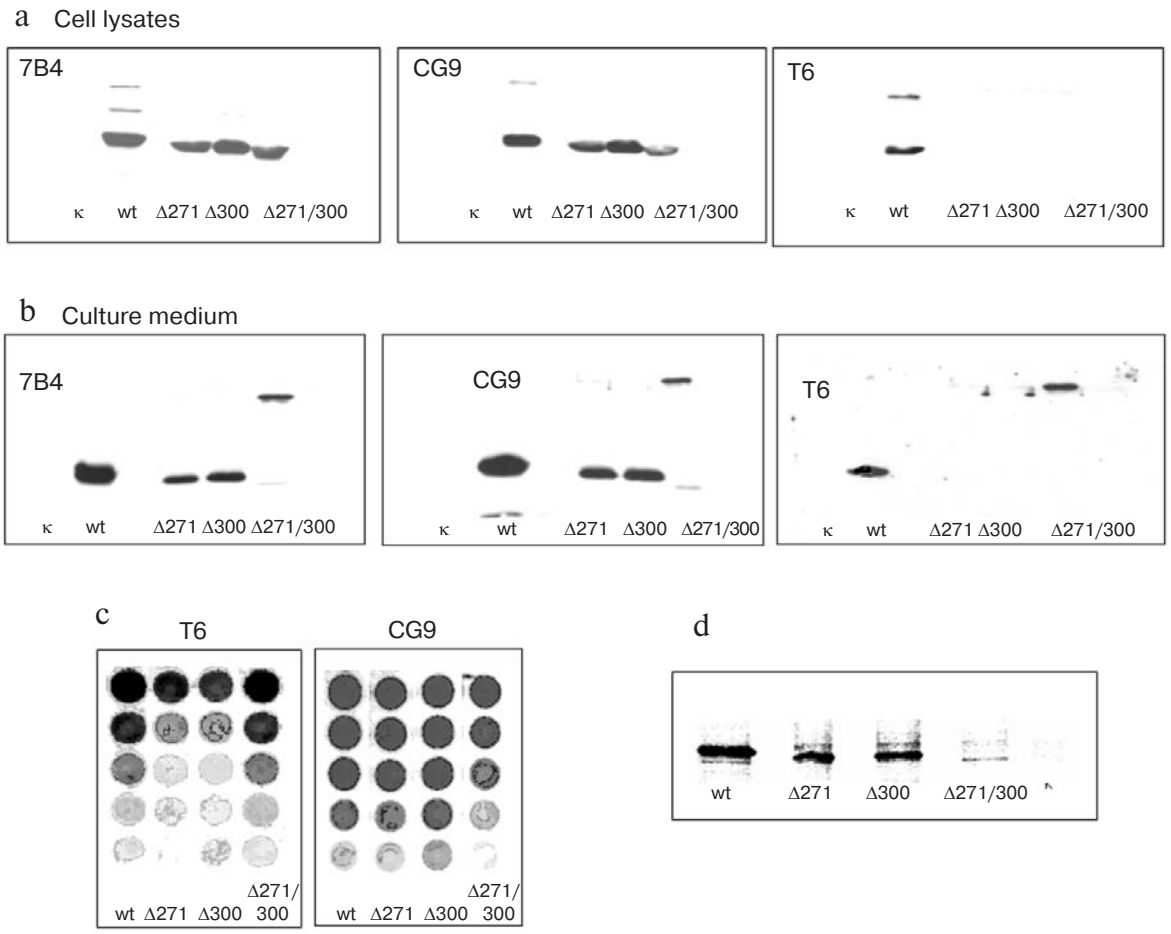

Fig. 5. Western blot and immunodot analyses of non-glycosylated sCD4. 293T cells were transfected with plasmids encoding wild type sCD4 (wt), Δ271, Δ300, and Δ271/300 mutants and with vector plasmid (k). In 18 h after the transfection, the media were exchanged for protein-free media, and 48 h after that the cells were lysed and the lysates were analyzed by Western blot (a). The growth media were also collected and analyzed by Western blot (b) and immunodot (c). d) Immunoprecipitation of ^{35}S-labeled CD4s by T6-Sepharose beads from the growth media of pulse/chased cells (see legend to Fig. 3).

causes such type of protein folding that leads to hiding of the T6 epitope. We note that the binding of other mAbs directed against the more membrane-distant D1-D2 domains is not affected by the presence or absence of the membrane anchor. We constructed a "tailed" form of CD4 where the first nine amino acids of the transmembrane sequence were added to the soluble form of CD4 (Fig. 1c). The 293T cells were transfected with the DNA construct. Expression of the "tailed" form was analyzed by *in situ* immunofluorescence staining and by Western blot in cell media (Fig. 6, a and b).

As can be seen, the "tailed" form was expressed and secreted similarly to the soluble form of CD4. However, change of Trp for Ser in position two of the tail sequence (see Fig. 1d) not influencing the intracellular expression (Fig. 6a) led to destabilization of D3/D4 domain conformation, as can be demonstrated by loss of recognition of such protein by mAb T6 in Western blot (Fig. 6b).

DISCUSSION

In this report, we show that sCD4 has a special conformation of D3/D4 domains that apparently does not exist in mCD4. However, the "natural" tail of sCD4 consisting of the first nine amino acids of transmembrane fragment of mCD4 was compatible with the correct folding. But changing the tryptophan residue for serine destabilized the D3/D4 conformation. Interestingly, tryptophan is found in many membrane-associated proteins in the same position [21-23]. In model experiments with synthetic transmembrane polypeptides, a preference for Trp for the anchoring of the peptide in the membrane was found [24-26]. It was proposed that the tryptophan side chain has a specific affinity for a well defined site near the interface between cell membrane and media and stabilizes the transmembrane peptide conformation [24]. The Trp residue probably separates the adjacent extracellular

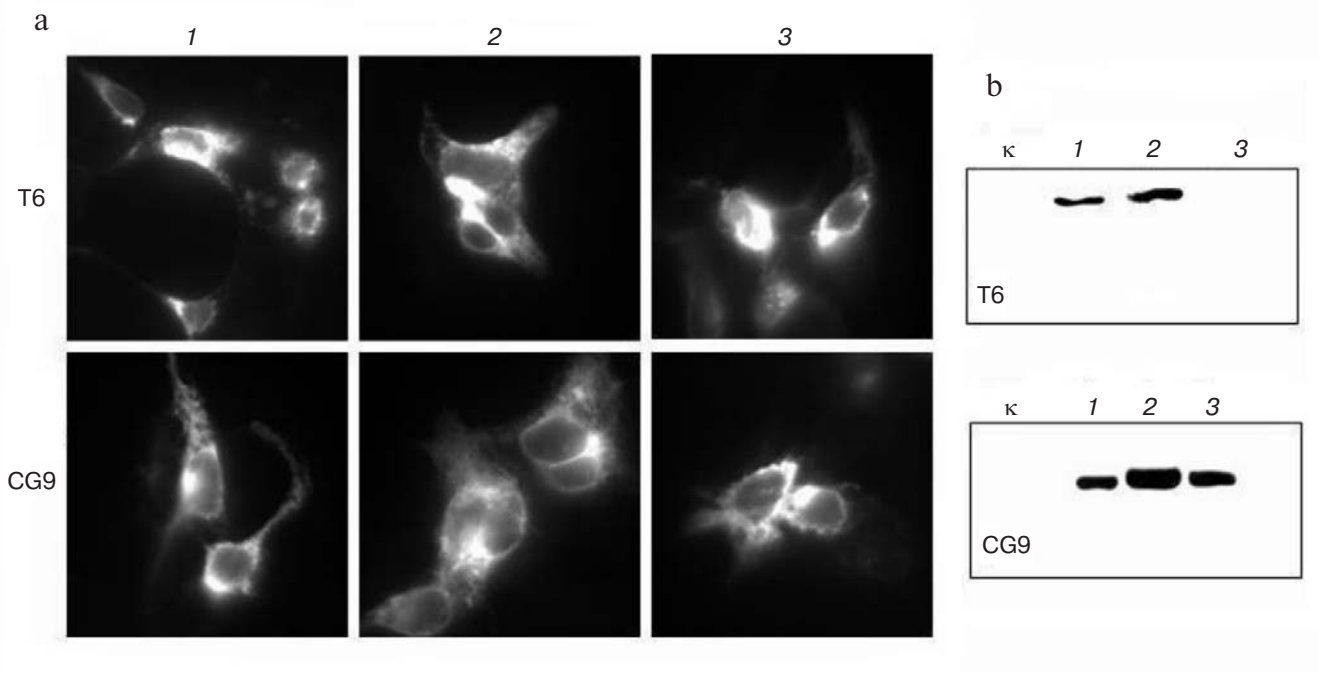

Fig. 6. Analysis of "extended" forms of sCD4. 293T cells were transfected with plasmids encoding sCD4 (*1*), "tailed" sCD4 (*2*), and W/S "tailed" sCD4 (*3*) or vector plasmid (k) on coverslips. a) At 48 h following transfection, the cells were treated with 3% *p*-formaldehyde and Triton X-100, incubated with mAbs CG9 or T6, and labeled with anti-mouse IgG mAb conjugated with FITC. Digital photography was performed using an imaging system on a Leica fluorescence microscope. The whole samples were analyzed and typical images are presented. b) The growth media of transfected 293T cells were run in 12% SDS-PAGE, transferred onto nitrocellulose membrane, and probed with mAbs T6 and CG9.

domain and transmembrane fragment of CD4. We note here that the introduction at the C-terminus of different "non-native" tails (data not shown) leads to hiding of T6 epitope, meaning that these tails play a role of folding inhibitors.

We found here that the epitope of T6 depends on protein sugar moieties, and the sugars not being involved in direct binding to the mAb stabilize the conformation of sCD4. While many of the conformational epitopes are readily restored in Western blot (under non-reducing conditions), refolding of sCD4 *in vitro* in the absence of sugar chains does not lead to restoration of the original, intracellularly created, conformation of D3/D4 domains of the protein. Probably the hydrophobic amino acid residues exposed on the molecular surface in the absence of sugars initiate *in vitro* in the absence of cellular chaperones a different type of protein folding.

In our experiments, both single glycosylation mutants of sCD4 were shown to be expressed and secreted by the cells. The Δ271/300 sCD4 double mutant was not detected intracellularly with mAbs T6 and CG76 (against D3/D4 domains), but after release into the media it becomes recognized by these mAbs. Probably epitopes of non-glycosylated D3/D4 region of the mutants are masked by non-lectin cellular chaperones binding hydrophobic polypeptide patches exposed in the absence of sugars.

We show here that the deglycosylated forms of sCD4 *in vivo* acquire a specific conformation similar to the wild type sCD4 which, however, cannot be restored after denaturation/renaturation under the conditions of non-reducing Western blot analysis. This observation indicates that the correct protein folding needs chaperone assistance and cannot be achieved *in vitro*.

In view of our data on competition between human anti-CD4 antibody and T6 mAb, we came to the conclusion that the anti-CD4 autoimmune response in HIV-1 patients is directed against the specific D3/D4 domain conformation not observed in native membrane-associated CD4. The sCD4 molecules were reported in the sera of HIV infected individuals [7]. Presumably, sCD4 may be part of the natural history of the disease. The human immune system normally should not respond to the soluble type conformation of CD4 because of the lack of T-help needed for the development of the efficient immune response. In the case of HIV infection, complexing of sCD4 with virus envelope protein gp120 can provide this help: gp120 being internalized together with sCD4 by sCD4-specific B-cells, will be presented in a complex with MHC class II molecule and recognized by gp120-specific T helper cells [27, 28].

Until recently, no functions have been attributed to the D3/D4 domains of CD4. But now data are published clearly indicating that domain 4 is involved in CD4

dimerization and required for interleukin (IL)-16 binding [29]. Subsequent studies showed that IL-16 is a multifunctional cytokine, selectively inducing the migration of CD4 T cells, eosinophils, and monocytes. IL-16 also acts as a growth factor for resting CD4 T cells, promoting their entry into the G1 phase of the cell cycle and inducing IL-2 receptor and MHC class II protein expression on the cell surface [29-33]. A direct interaction between IL-16 and CD4 was observed in co-immunoprecipitation experiments [29]. Therefore, the demonstration of the D3/D4 domain flexibility could contribute to our understanding of CD4 functioning.

The mechanism of sCD4 release is unknown, but it is likely that it involves proteolytic cleavage of a membrane-anchored protein at a site close to the cell surface. Alternative mRNA splicing might also lead to the expression of soluble forms of proteins, which lack cytoplasmic and membrane-spanning domains of the membrane-associated protein. However, in a number of cases where sCD4 is detected in serum, "anchor minus" cDNA has not been found [8, 23, 34].

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