Crystallization of a Deglycosylated T Cell Receptor (TCR) Complexed with an Anti-TCR Fab Fragment*  

(Received for publication, August 28, 1996, and in revised form, October 9, 1996)

Ju Liu‡§, Albert G. D. Tse¶¶, Hsiu-Ching Chang‡§, Jin-huan Liu‡§, Jiahuai Wang‡¶¶, Rebecca E. Hussey‡¶¶, Yasmin Chishti‡¶, Bruce Rheinhold**‡, Rebecca Spoerl‡, Stanley G. Nathenson‡‡, James C. Sacchettini§§, and Ellis L. Reinherz‡§¶¶

From the ‡Laboratory of Immunobiology, Dana-Farber Cancer Institute and the Departments of §Medicine and ¶Pathology, Harvard Medical School, Boston, Massachusetts 02115, the **Department of Biophysics, Boston University Medical School, Boston, Massachusetts 02118, and the Departments of ¶¶¶Microbiology and Immunology and §§Biochemistry, Albert Einstein College of Medicine, Bronx, New York 10461

A strategy to overexpress T cell receptors (TCRs) in Lec3.2.8.1 cells has been developed using the “Velcro” leucine zipper sequence to facilitate α–β pairing. Upon secretion in culture media, the VSV-8-specific/H2-Kb-restricted N15 TCR could be readily immunoprecipitated using the anti-leucine zipper monoclonal antibody 2H11, with a yield of 5–10 mg/liter. Mass spectrometry analysis revealed that all attached glycans were GlcNAc2-Man1. Following Superdex 200 gel filtration to remove aggregates, wild-type N15 or N15a, a C183S variant lacking the unpaired cysteine at amino acid residue 183 in the Cβ domain, was thrombin-cleaved and endoglycosidase H-digested, and the two derivatives were termed iN15ΔH and N15ΔH, respectively, and sized by Superdex 75 chromatography to high purity. N-terminal and C-terminal microsequencing analysis showed the expected unique termini of N15 α and β subunits. Nevertheless, neither protein crystallized under a wide range of conditions. Subsequently, we produced a Fab fragment of the murine TCR Cβ-specific hamster monoclonal antibody H57 and complexed the Fab fragment with iN15ΔH and N15ΔH. Both N15ΔH-Fab[H57] and iN15ΔH-Fab[H57] complexes crystallize, with the former diffracting to 2.8 Å resolution. These findings show that neither intact glycans nor the conserved and partially exposed Cys-183 is required for protein stability. Furthermore, our results suggest that the H57 Fab fragment aids in the crystallization of TCRs by altering their molecular surface and/or stabilizing inherent conformational mobility.

The TCR complex consists of multiple transmembrane polypeptide chains on the surface of T lymphocytes (1–3). The disulfide-linked αβ heterodimer (Ti) is the clonally unique component that possesses a recognition site for antigen in the context of a major histocompatibility complex protein (4, 5). Sequence analysis of α and β subunits strongly supports that their ectodomains form a recognition unit reminiscent of an immunoglobulin Fab fragment (6–8). This notion has been confirmed in crystallographic studies of TCR subunit fragments (9, 10). On the other hand, the invariant CD3 components (%, $, c, $, and q) possess lengthy cytoplasmic tails containing immune cell tyrosine-based activation motifs and are involved in signal transduction (11, 12). To date, most of the attributes of TCR recognition have been studied largely indirectly because of the intimate membrane association of this complex.

To understand the process by which T cells recognize pathogens in explicit molecular terms, recent efforts have begun to focus on the structural nature of the TCR. However, many efforts to express soluble TCR αβ heterodimers in both prokaryotic and eukaryotic systems have been hampered by inefficient pairing of α and β subunits in the absence of their respective transmembrane regions and associated CD3 components (reviewed in Refs. 13–21). We have recently developed a methodology to overcome this obstacle by adding 30-amino acid-long segments to the carboxyl termini of α and β extracellular domains via a thrombin-cleavable flexible linker (22). These peptide segments (Base-p1 for α and Acid-p1 for β) were previously shown to selectively associate to form a stable heterodimeric coiled coil termed the leucine zipper (23). Homodimeric structures are not favored due to the electrostatic repulsion among amino acid side chains. Furthermore, the yield of these engineered proteins was 5–10-fold greater than that of the TCR expressed in the absence of the synthetic leucine zipper (22). Through the use of a panel of mAbs directed at native α and β epitopes within constant and variable regions, it was further shown that the fusion heterodimer was native.

Efforts to obtain TCR crystals from such material, however, were unsuccessful, perhaps owing to the glycosylation heterogeneity inherent in the baculovirus system and/or the intrinsic conformer heterogeneity of the TCR heterodimers under examination. Here we describe a system using Chinese hamster ovary Lec3.2.8.1 cells (24) to express TCRs that can be readily deglycosylated by endoglycosidase H. Our results demonstrate that deglycosylation of the TCR with endoglycosidase H does not alter the native TCR structure, implying that the intact glycans are not required for the protein’s stability. The behavior of a C183S TCR variant lacking the unpaired cysteine at amino acid residue 183 of the Cβ domain indicates that this partially exposed and conserved residue is not necessary for TCR expression or structure. We further show that by complexing such a deglycosylated TCR derivative with a specific anti-
TFR Fab fragment, it is possible to obtain TCR crysalts that diffract at atomic resolution.

**EXPERIMENTAL PROCEDURES**

**Cloning of N15 into the pEE14-GS Expression Vector**—The generation of constructs containing the TCR fused to leucine zipper component was performed essentially as described previously (22). In brief, two DNA fragments encoding a linker sequence and leucine zipper peptide were generated using synthetic oligonucleotides and polymerase chain reaction. The DNA fragments were then subcloned into the BamHI-EcoRI site of the pCR2 vector (Invitrogen) for DNA sequencing analysis and further manipulation. The TCR zipper cDNA was then ligated to the N15Acid plasmid vector. The pEE14-N15Acid plasmid was then digested with MluI and released the DNA fragment containing the cDNA of N15Acid promoter plus N15Acid. The DNA fragment was then subcloned into MluI site of pEE14-N15Acid to yield the full-length DNA fragment encoding TCR fusion protein.

**Expression of N15 Using a Glutamine Synthetase Vector in Lec3.2.8.1 Cells**—The Lec3.2.8.1 cells were grown at 37°C as a monolayer in a minimal essential medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum, 2 mM glutamine, and 50 units/ml penicillin/streptomycin.

**Large-scale Production of TCR Protein**—The transfected Chinese hamster ovary (CHO) cells were harvested using 0.125% trypsin and 0.5 mM EDTA and pelleted for 10 min at 2000 rpm. The cells from the remaining 20 flasks were harvested using 0.125% trypsin and 0.5 mM EDTA and expanded into 50 175-cm² tissue culture flasks (Falcon) in 35 ml of complete GMEM-S medium and were grown to confluence (~3–4 days). Subsequently, an additional 35 ml of fresh GMEM-S medium containing 5% dialyzed fetal calf serum, 25 µg/ml methionine sulfoximine, and 4 mM sodium butyrate was added to each of 40 flasks, and cells were cultured for an additional 5 days, which time the supernatants were harvested and centrifuged for 10 min at 2000 rpm. The cells from the remaining 20 flasks were harvested using 0.125% trypsin and 0.5 mM EDTA and expanded into 50 175-cm² tissue culture flasks, and the entire process was repeated.

**Purification of sTCR Protein**—The Lec3.2.8.1 supernatants of N15–2B3 and N15–S29 containing N15 sTCR-leucine zipper fusion proteins (N15 and N15*, respectively) were filtered using a Corning filter (0.22 µm), and further steps were performed at 4°C. N15 and N15* were purified by a two-step procedure involving immunoaffinity chromatography and gel filtration. The first step used mAb 2H11 coupled at 5 mg/ml to GammaBind Plus-Sepharose beads (Pharmacia) via cross-linking with dimethyl pimelimidate, and the second step consisted of FPLC (Pharmacia) gel filtration chromatography on a Superdex 200 column controlled by a computer FPLC director program (Pharmacia). Approximately 15 liters of N15 supernatants was first passed over the GammaBind Plus-Sepharose bead precolumn (5-mL bed volume), followed subsequently by a mAb 2H11 affinity column (also 5-mL bed volume) using pump (Bio-Rad) control with a flow rate of ~0.5 mL/min. To remove any contaminating proteins bound nonspecifically to the mAb 2H11 resin, an extensive washing step was performed with a pH step gradient from pH 7.5 to pH 6.8 buffer (20 mM Tris/acidic (pH 6.8), 0.5 mM NaCl, and 5% glycerol) using at least 50 volumes the column bed volume. The efficiency of the washing was monitored by observing the A280 of the flow-through supernatant, with washing continued until the absorbance fell below 0.020. Next, the bound N15* was eluted with low pH buffer (50 mM citrate, 20 mM Tris, 0.5 mM NaCl, and 5% glycerol (pH 4.0)), and peak fractions were immediately adjusted to pH 7.2 using 1 × Tris-HCl (pH 9.5), pooled, and dialyzed against TBS (20 mM Tris and 150 mM NaCl (pH 7.2)) with 10 mM EDTA overnight at 4°C. The purified protein was concentrated to 2 mL with an Amicon Centriprep-10 concentrator and loaded onto a 1.6 × 60-cm Superdex 200 column (Pharmacia) equilibrated with TBS.

**Microchemical Analysis of Purified Proteins**—Protein concentration of N15 samples was determined using the specific extinction coefficient at 280 nm (0.69), which was calculated by the method of Gill and von Hippel (26), as well as by using either the Bio-Rad version of the Bradford assay for binding the micro-BCA protein assay (Pierce). Amino-terminal microsequencing was carried out on 450 pmol of N15 protein on an Applied Biosystems Model 477A pulsed liquid-phase protein sequencer. For C-terminal amino acid sequencing, we employed a Hewlett-Packard C-terminal sequencer using the C-terminal routine 2.0 with the samples spotted on a Zytex membrane. Protein analysis was performed on denaturing gels using 10 or 12% SDS-PAGE by the Laemmli method (27). Prior to electrophoresis, nonreducing samples were heated at 65°C for 10 min or at 100°C for 5 min, and reducing samples were boiled for 5 min in a loading buffer containing 100 mM diethiothreitol. Gels were stained with Coomassie Brilliant Blue R-250, and the protein bands were quantitated by laser densitometry (Pharmacia Biotech Inc.). Isoelectric focusing (IEF) of native gels was carried out using a PhastSystem using precast gels (pH range 3–9) (Pharmacia), while native gels were 8–25%.

**Glycan Analysis by Electro spray Ionization Mass Spectrometry**—For mass spectrometry, the purified N15 protein was treated with endoglycosidase H (endo-H) (Boehringer Mannheim) following the manufacturer’s instructions. The released glycans and protein was methylated, which also releases and methylates possible O-linked glycans (28, 29).
The methylated glycans were chloroform-extracted, dried under vacuum solution in a methanol/water solution (84:16, v/v) containing 0.25 M sodium hydroxide. Electrospray solutions were injected into the mass spectrometer at a rate of 0.75 liter/min through a stainless steel hypodermic needle. The voltage differences between the needle tip and the source electrode were 3–4 kV. For collision-induced dissociation studies, singly or multiply charged precursor ions were selectively transmitted by the first mass analyzer and directed into the collision cell containing argon at roughly 2 millitorrs with acceleration voltages of 20–60 V, and the fragments were scanned in a quadrupole mass analyzer after the collision cell as described (28).

**Immunoreactivity Analysis**—The reactivities of N15 and endo-H-treated N15 (N15b) with a panel of mAbs were examined using a sandwich enzyme-linked immunosorbent assay procedure. In brief, 5 μg/ml N15 or H57 was coated on Immulon-II plates at room temperature for 2 h and blocked with 1% bovine serum albumin in borate-buffered saline (pH 8.3) at room temperature for 2 h. Then, 50 ng of N15 or N15b was added to each well overnight at 4 °C. Subsequently, 50 μl of various hybridoma cell supernatants was added for 2 h at room temperature, and the reaction was developed with horseradish peroxidase-conjugated secondary antibodies. Each comparison of a mAb reactivity with N15 and N15b was expressed as the ratio of A405 with N15b to A405 with N15.

**Preparation of TCR Derivatives**—Any potentially exposed free sulfhydryl group of Cys-183 in the β-chain domain was blocked either with N-ethylmaleimide or iodoacetamide. Using [14C]iodoacetamide (Amer sham Corp.), radioactivity was found to be incorporated into the β chain in H57 incubated with 10 mM HEPES, 10 mM Tris, and 100 mM NaCl (pH 8.0) after 2 h of incubation at 27 °C (data not shown). Since both chemical modifications resulted in proteins with similar crystallographic behavior, only the iodoacetamide results are presented herein. Modified N15 (iN15) and N15b were digested with bovine thrombin (Calbiochem) at 27 °C in HEPES buffer for 3 h (10–20 mg/500 units of enzyme) to yield IN15a and IN15b, respectively. For iodoacetamide modification, the chemical (1:10 molar ratio) was added just before thrombin so that blockage and cleavage were carried out at the same time. Subsequently, after the protein solution was buffer-exchanged into 50 mM NaAc (pH 5.5), the protein was incubated at room temperature, and the reaction was developed with horseradish peroxidase-conjugated secondary antibodies. Each comparison of a mAb reactivity with N15 and N15b was expressed as the ratio of A405 with N15b to A405 with N15.

**Generation of the H57 Fab Fragment**—Forty to one-hundred milligrams of H57 antibody was purified from Cell Pharm supernatants on a Superdex S-75 column (1.6 cm × 60 cm; Pharmacia) in 150 mM NaAc at 0.5 mM/min. The column was run at 4 °C under the control of PFFC, and 84 × 1 ml fractions were collected after 36 ml. An aliquot of the peak fractions was analyzed by nonreducing SDS-PAGE.

**RESULTS AND DISCUSSION**

**Baculovirus-produced N15 Glycoproteins Are Resistant to Endoglycosidase H Treatment**—Prior studies showed that expression of a representative TCR-leucine zipper fusion protein (N15) in a baculovirus system yielded the desired TCR αβ heterodimers without formation of unwanted αα or ββ homodimers (22). Although expression was adequate at 1 mg/liter and purity >90%, the N15 protein was unable to crystallize under a wide range of experimental conditions (data not shown). Because the β chain was variably glycosylated, we attempted to remove N-linked glycans by treatment of the purified baculoN15 protein with endo-H assuming that glycan heterogeneity was detrimental to the crystallization efforts. As shown in Fig. 1A, however, even at an enzyme/substrate ratio of 5 μg of protein to 1 milliliters of endo-H for a 16-h incubation, baculoN15 was resistant to endo-H digestion. This resistance of the baculoN15 glycans is not unexpected due to core fucosylation of a large fraction of the oligomannose oligosaccharides in the baculovirus expression system (32, 33). BaculoN15 was also screening crystallization conditions. Typically, ~100 different conditions were tried for each protein sample. The most promising condition was then refined for growing crystals of diffraction quality.

**X-ray diffraction pictures were taken of a cluster of crystals (dimension of ~100 × 50 × 10 μm) using an Elliot GX-13 rotating anode generator operated at 40 kV and 60 mA for CuKα radiation. This was very useful for testing the diffraction behavior of various crystals obtained and permitted the identification of a suitable cryoprotectant buffer for freezing the crystals at −160 °C. A 2.8-Å data set was collected on the X12C beamline at the National Synchrotron Light Source Brookhaven National Laboratory with a single frozen crystal of 150 × 100 × 25 μm under −160 °C on a MAR image plate.

**RESULTS AND DISCUSSION**

**Baculovirus-produced N15 Glycoproteins Are Resistant to Endoglycosidase H Treatment**—Prior studies showed that expression of a representative TCR-leucine zipper fusion protein (N15) in a baculovirus system yielded the desired TCR αβ heterodimers without formation of unwanted αα or ββ homodimers (22). Although expression was adequate at 1 mg/liter and purity >90%, the N15 protein was unable to crystallize under a wide range of experimental conditions (data not shown). Because the β chain was variably glycosylated, we attempted to remove N-linked glycans by treatment of the purified baculoN15 protein with endo-H assuming that glycan heterogeneity was detrimental to the crystallization efforts. As shown in Fig. 1A, however, even at an enzyme/substrate ratio of 5 μg of protein to 1 milliliters of endo-H for a 16-h incubation, baculoN15 was resistant to endo-H digestion. This resistance of the baculoN15 glycans is not unexpected due to core fucosylation of a large fraction of the oligomannose oligosaccharides in the baculovirus expression system (32, 33). BaculoN15 was also

![Fig. 1. Endoglycosidase H digestion of the N15 TCR protein derived from insect high five cells (baculoN15) and from the Loc32.2.5.1 cell line (N15). Fifty micrograms of purified baculoN15 or N15 was incubated at room temperature in endoglycosidase H (10 milliunits). Aliquots were taken at time 0 or after 30 min, 3 h, and 16 h of digestion, and products were analyzed by 10% SDS-PAGE. In A, all samples were examined under nonreducing conditions. In B, NR = nonreducing conditions, and + and − refer to the presence or absence of endo-H, respectively. C shows the pEE14GS-N15Loc32.2.5.1 expression plasmid containing the N15 α and β subunits fused to Base-p1 and Acid-p1 leucine zipper peptides.](image-url)
resistant to enzymatic deglycosylation with N-glycan as well (data not shown).

Expression of the N15 TCR in Lec3.2.8.1 Cells—To obtain a form of TCR protein from which the glycan could be readily cleaved, we expressed the N15 TCR in the Chinese hamster ovary Lec3.2.8.1 cell derivatives that exclusively synthesize homogeneous high mannose glycans (24). For this purpose, the same N15 TCR α and β cDNAs encoding 30-amino acid-long leucine zipper sequences appended to the carboxyl termini of the α and β extracellular domains (via a flexible linker with a thrombin cleavage site) as used in the baculovirus vector were cloned into the pEE14-GS vector as pEE14GS-N15αBase and pEE14GS-N15βAcid, respectively and then to create pEE14GS-N15β (Fig. 1C). The latter was transfected into Lec3.2.8.1 cells, and clones were selected by screening cell supernatants for immunoreactivity with anti-TCR Cα and Cβ mAbs or the anti-leucine zipper mAb 2H11 as described under “Materials and Methods.” In this way, Lec3.2.8.1 clones producing N15 αβ heterodimers (N15) at 5–10 mg/liter were obtained.

As shown in Fig. 1 (A and B), the N15 protein, in contrast to baculoN15, could be readily deglycosylated after just a 30-min exposure to endo-H under the same experimental conditions used for baculoN15. SDS-PAGE analysis indicated that under nonreducing conditions, the size of the glycosylated heterodimer shifts from 80–90 to 60–70 kDa upon endo-H treatment. Moreover, under reducing conditions, the endo-H treatment result in a shift of the N15 α band from 36 to 31 kDa and collapse of the three N15 β bands at 41, 43, and 45 kDa to a single 39-kDa band. Note that protein sequence analysis of baculoN15 previously demonstrated that the closely spaced and more slowly migrating bands in the gel were N15 β-related and that the single rapidly migrating band corresponded to N15 α (Ref. 22 and data not shown). Removal of several N-linked glycans on each N15 subunit could account for this shift in apparent molecular mass. Potential N-linked glycosylation sites are found in the N15 α subunit at Asn-21, Asn-179, and Asn-193 and in the N15 β subunit at Asn-77, Asn-117, Asn-179, and Asn-228. Hence, distinct glycoforms of the αβ heterodimer with variable occupancy of the N-linked sites on the β chain are consistent with the SDS-PAGE analysis. However, if the glycan distribution were random and the resolution of the gel system sufficient, we would expect to detect five β bands (corresponding to β chains with 0–4 occupancy of the N-linked sites). Given that there are only three β bands visualized, a subset of these conformers/glycoforms is present. Such preferential sitespecific glycosylation has been reported previously for other proteins (34). Because α subunit Asn-179 and β subunit Asn-117 are predicted to lie in close proximity to one another on the surface of N15,2 it is unlikely that both sites will be glycosylated on the same molecule. That three discretely sized β bands but only one α band exist as indicated by reducing SDS-PAGE analysis implies that glycosylation of the N15 β subunit is more heterogeneous than that of the α subunit.

Electrospray Ionization Mass Spectrometry Analysis of N15 Glycans—To examine whether the heterogeneity in the N15 β chain resulted from differential utilization of potential N-linked glycosylation sites on individual N15 β chains and/or heterogeneity in the nature of the glycans attached to the various N-linked sites, mass spectrometry analysis was performed on the endo-H-cleaved N15 glycan (Fig. 2). Electrospray mass spectrometry ionizes methylated carbohydrates by the addition of alkali metal cations added to the solution. The m/z values of 1335 and 679 in the electrospray spectrum (Fig. 2A) correspond to a molecular species of 1312 Da adducted with one and two sodium cations, respectively. A molar mass of 1312 Da is the expected mass of a methylated oligosaccharide with five hexose (mannose) residues and a single N-acetyllactosamine (N-acetylglucosamine) residue.

Collision-induced dissociation of methylated and alkali metal-adducted oligosaccharides was also performed (Fig. 2B) to identify high mannose branching isomers by a combination of glycosidic fragments, which convey information on the connection topology of the mannose residues, and ring opening fragments, which identify specific linkages. The lack of an ion fragment pair at m/z 445 (charge on the nonreducing end) and 912 (charge on the reducing end) excludes topologies in which two mannose residues are eliminated by cleaving a single glycosidic bond. The ion pair at m/z 709 (overlapped with a m/z 708 reducing end fragment) and 737 arises from dissociation of the core mannose pyranose ring and identifies a 6-linked branch containing three hexose residues. These structural features identify the specific Man_{n} isomer shown in Fig. 2B as the only glycan on the N15 TCR. Hence, the differences in mobility of N15 β subunits under reducing SDS-PAGE must be a consequence of differential utilization of N-linked sites. Given that the protein/glycan mixtures were also methylated and would have released methylated O-linked glycans, the lack of detectable O-linked glycan adducts of N15 α or β subunits implies that the N15 sTCR undergoes no O-linked glycosylation in Lec3.2.8.1 cells.

Unaltered Immunoreactivity of N15, Compared with That of N15—To examine whether endo-H treatment of the N15 protein (N15_,H) altered its immunoreactivity relative to non-deglycosylated N15, a comparative enzyme-linked immunosorbent assay analysis (see “Materials and Methods”) was performed using the previously described anti-Cβ mAb H57 (35), anti-V65.2 mAb MR9.4 (36), and N15 Vβ-reactive mAbs N15R4, R7, R8, R13, R15, R22, R28, R34, R33, R46, R53, and R54 (22). Among the V65.2-specific mAbs, N15 R53 is clonotypic, being directed at the CDR3 region of the N15 β chain. When each of

---

1 J. Liu, A. G. D. Tse, H.-C. Chang, J.-h. Liu, J. Wang, R. E. Hussey, Y. Chishti, B. Rheinhold, R. Spoerl, S. G. Nathenson, J. C. Sacchettini, and E. L. Reinherz, unpublished data.

2 J. Liu, A. G. D. Tse, H.-C. Chang, J.-h. Liu, J. Wang, R. E. Hussey, Y. Chishti, B. Rheinhold, R. Spoerl, S. G. Nathenson, J. C. Sacchettini, and E. L. Reinherz, unpublished data.
Because we suspected that the unpaired cysteine at residue 183 within the β chain constant region might create disulfide-linked N15 dimers and further complicate biochemical purification by fostering disulfide exchange, we mutated this cysteine to a serine, the corresponding residue found in Ig C\textsubscript{H1} domains (37). cDNAs encoding the mutated TCR (N15\textsuperscript{s}) were then expressed in Lec3.2.8.1 cells. Fig. 3A shows that the chromatographic profile of N15\textsuperscript{s} is very similar to that of N15. However, there is a substantial reduction in disulfide-linked N15 dimers observed by SDS-PAGE under nonreducing conditions (Fig. 3B). Moreover, the amount of non-disulfide-linked heterodimer as judged by nonreducing SDS-PAGE is dramatically reduced (Fig. 3B). Because attempts to crystallize N15 and N15\textsuperscript{s} proteins under a range of conditions were unsuccessful, we next generated a series of TCR derivatives and assessed whether they were more suitable crystallization candidates.

**Generation and Purification of TCR Derivatives**—To generate sTCR derivatives, both N15 and N15\textsuperscript{s} were utilized as sources of starting proteins. In the case of N15, the accessible SH group of β chain Cys-183 was derivatized with iodoacetamide (iN15) to prevent disulfide exchange. N15 and N15\textsuperscript{s} were thrombin-cleaved to yield iN15\textsubscript{A} and N15\textsubscript{A}\textsuperscript{ΔH}, respectively, or additionally digested with endo-H, resulting in iN15\textsubscript{A} and N15\textsubscript{A}\textsuperscript{ΔH}, respectively. From the overlay of chromatograms depicted in Fig. 4A, it is obvious that the double-digested iN15\textsubscript{A} and N15\textsubscript{A}\textsuperscript{ΔH} derivatives have a smaller hydrodynamic volume than the thrombin only-digested iN15\textsubscript{A} and N15\textsubscript{A}\textsuperscript{ΔH} derivatives, with \(V_e = 57.7\) ml versus 55.5 ml, respectively. When the fractions across the major peaks were analyzed by nonreducing SDS-PAGE (Fig. 4B), we observed protein bands above the major one spreading from the earlier fraction into the main peak; this was most evident in the iodoacetamide-treated derivatives iN15\textsubscript{A} and iN15\textsubscript{A}\textsuperscript{ΔH}. Furthermore, there were non-disulfide-linked heterodimers comigrating with the disulfide-linked equivalents in the iN15\textsubscript{A} and iN15\textsubscript{A}\textsuperscript{ΔH} samples. On the other hand, the overlay of the chromatograms in Fig. 4A indicates that the hydrodynamic properties of the N15 and N15\textsuperscript{s} proteins were similar after comparable treatment. More important, as shown in Fig. 4B, the overall complexity of the N15\textsuperscript{s} derivatives was less, including virtual elimination of the non-disulfide-linked heterodimers.

The N termini of the N15\textsubscript{A}\textsuperscript{ΔH} heterodimeric protein were sequenced through amino acid residue 14; signal(s) generated at each cycle matched the predicted N15 α and β sequences. This result shows that there have not been any unexpected proteolytic events in the TCR subunits during thrombin cleavage and/or endo-H digestion. Furthermore, C-terminal sequencing of N15\textsuperscript{A}\textsubscript{ΔH} unambiguously defined arginine in cycle 1, consistent with the fact that thrombin cleaves within the flexible linker regions of each subunit after arginine and before glycine. In agreement with this result, mass spectrometry analysis of the leucine zipper peptide showed a mass (1373.3 and 1375.9 triple charged state) essentially identical to that of the expected cleavage product (1373.7 and 1376.2) with no detectable heterogeneity. The various N15 and N15\textsuperscript{s} derivatives each underwent a crystallization trial. However, none of these sTCR derivatives yielded crystals.

**pI Heterogeneity of TCR Derivatives**—The above result is interesting in view of the lack of N- or C-terminal heterogeneity in N15\textsubscript{A}\textsuperscript{ΔH} and the homogeneous size of the N15\textsubscript{A}\textsuperscript{ΔH} protein in the peak fractions as assessed by SDS-PAGE (Fig. 4B, panel d). To address whether there might be heterogeneity within the TCR components by other criteria, native IEF gels were run, and the various TCR derivatives were compared on the basis of charge. As shown in Fig. 5, at least three major pI bands and several minor bands are observed within the 6.5–8 pI range for...
TCR αβ Heterodimer Crystals

N15 and N15s. Moreover, this charge heterogeneity is observed in purified N15ΔΔ, indicating that the heterogeneity is not a function of the leucine zipper sequence, but rather is intrinsic to the TCR itself. However, note that removal of the leucine zipper sequence results in a shift of the protein pI to the TCR itself. However, note that removal of the leucine zipper sequence, but rather is intrinsic. The smaller peak eluting at ~76 ml represents the cleaved Velcro sequences. Other minor peaks (digested fragments) eluting after 80 ml are not shown for clarity. Four to eight microliters from each fraction around the main peak (B: panel a, fractions 18–28; panel b, fractions 18–28; panel c, fractions 14–25; panel d, fractions 17–28) was analyzed by 12% nonreducing SDS-PAGE (except where indicated) and stained with Coomassie Blue. Orig., original intact N15; orig.H, time 0 sample (after thrombin digest) before endo-H digestion; N15R, original N15 reduced; 20R, fraction 20 reduced; 23R, fraction 23 reduced.

Several explanations might account for this rather striking charge heterogeneity. One possibility is that the N15 TCR can exist in several stable conformational states, perhaps reflecting variability in the quaternary structure of the heterodimer. Alternatively, differential occupancy of N-linked glycosylation sites (with GlcNAc-2-Man for N15 or with GlcNAc for N15ΔΔ) on the β subunits may alter the surface charge within the N15 TCR preparation. This possibility seems more remote, however, in view of the similar extent of heterogeneity when comparing N15ΔΔ and N15ΔH, given that only one GlcNAc is attached to the N-linked sites of the latter. Furthermore, that pI heterogeneity is not unique to the N15 TCR is clear from analysis of the unrelated N26 TCR specific for VSV-S/Kb (data not shown).

Complex Formation between sTCR Derivatives and the H57 Fab Fragment—Given the failure of N15 TCR proteins to yield useful crystals on their own and in view of several independent observations (38) showing that Fab fragments can stabilize proteins by offering new molecular surfaces for crystallization, we next complexed N15, N15s, and their derivatives with the H57 Fab fragment. The hamster H57 mAb is specific for the mouse Cβ TCR constant region. To this end, the Fab fragment was generated by immobilized papain, purified, and tested by titration analysis with the TCR on an 8–25% native Phastgel to observe a complex formation. By maintaining the Fab fragment amount constant and titrating in an increasing volume of N15ΔΔ, it can clearly be seen that a new band forms corresponding to the N15ΔΔFab complex (data not shown). In representative experiments, full complexation is evident at an N15ΔΔFab ratio of 3:1 (ν/ν) since neither Fab nor N15ΔΔ bands are detected. TCR excess occurs as the amount of N15ΔΔ increases relative to the Fab fragment, e.g. at a ratio of 4:1 or higher.

Crystallization of TCR Derivatives with the H57 Fab Fragment—Once the ratios of TCR to the Fab fragment giving a molar complex formation were determined, large-scale mixing of TCR and the Fab fragment was performed. The TCR-Fab complex was then concentrated to 10–13 mg/ml, and crystallization trays were set up. Table I summarizes the results of the crystallization trials. Essentially, the H57 Fab fragment complexes with all of the TCR from Lec3.2.8.1 cells, whether they are wild-type or mutant, with zipper or without; thrombin cleavage or thrombin cleavage plus endo-H digestion forms crystals under very similar conditions. Typically, the crystallization buffer contained 8–10% polyethylene glycol 8000, 0.2 M KCl, and 0.1 M acetate (for pH 5.5) or cacodylate (for pH 6.5) or HEPES (for pH 7.0). In the case of the H57 Fab complex with N15, iN15Δ, N15Δs, and N15ΔH, the crystals were too small in size and too heavily clustered to be suitable for x-ray work. In contrast, iN15Δ9-Fab[H57] appeared as well developed hexagonal rods reaching a size of ~1 mm long and 400–500 µm across. Unfortunately, these crystals give diffraction to only a 9-Å resolution. More important, the N15ΔΔH-Fab[H57] complex
yielded crystal growth over quite a broad pH range (5.5–7.0). These crystals tend to cluster. At low pH, the flower-like fine crystals appear first; later clusters of wedge-shaped single crystals are produced. To date, the largest crystals measure 400 × 200 × 30 µm. These crystals demonstrate a strong polarization of light. Single crystals can diffract to beyond 2.8 Å, with crystal structure, with silver staining of both these crystals confirmed the presence of intact glycans nor the invariant unpaired Cys-183 in the Cα domain stabilizes the TCR heterodimer despite a weak intrinsic association between α and β subunits.

The rather striking pI heterogeneity of the TCR αβ derivatives even in the absence of glycan uncovered herein is most likely a reflection of different TCR conformers. Such conformational mobility may arise from floppiness within the α subunit itself or at the interface between Vα and Vβ or Ca and Cβ modules. This potential mobility may be important in TCR signal transduction. Our results further indicate that neither intact glycans nor the invariant unpaired Cys-183 in the Cβ domain is required for stability of the TCR structure.

Finally, from our current results, we conclude that altering the TCR molecular surface and/or restricting the mobility of TCR conformers through ligation of the Fab fragment to the TCR is important for crystallization of TCRs such as N15. If this notion is correct, then ligation of a TCR with antigen/major histocompatibility complex or a superantigen may offer alternative strategies capable of accomplishing the same goal. From a practical perspective, the use of a Fab fragment specific for all TCR β constant regions may be a useful way to overcome crystallization difficulties with TCRs alone in situations where superantigen binding or peptide/major histocompatibility complex binding is not feasible.

Acknowledgments—We thank Robert Sweet and the staff of the X12C beamline at Brookhaven National Laboratory for support and encouragement.

REFERENCES

1. Meuer, S. C., Acuto, O., Herend, T., Schlossman, S. F., and Reinherz, E. L. (1984) Annu. Rev. Immunol. 2, 23–59
2. Ashwell, J. D., and Klausner, R. D. (1990) Annu. Rev. Immunol. 8, 139–167
3. Davis, M. M., and Bjorkman, P. J. (1988) Nature 334, 385–402
4. Meuer, S. C., Acuto, O., Hussey, R. E., Hodgson, J. C., Fitzgerald, K. A., Schlossman, S. F., and Reinherz, E. L. (1993) Nature 363, 808–810
5. Haskins, K., Kubo, R., White, J., Pigeon, M., Kappler, J., and Marrack, P. (1983) J. Exp. Med. 157, 1119–1138
6. Novotny, J., Tonegawa, S., Hsiao, C., Kranz, D., and Eisen, H. N. (1986) Proc. Natl. Acad. Sci. U.S. A. 83, 742–746
7. Chothia, C., Boswell, D. R., and Lesk, A. M. (1988) EMBO J. 7, 3745–3755
8. Claverie, J.-M., Prochnicka-Chalouf, A., and Bouguerelet, L. (1989) Immuno- nol. Today 10, 10–14
9. Bentley, G. A., Boullet, G., Karjalainen, K., and Mariuzza, R. A. (1995) Science 267, 1984–1987

Table I

Summary of recombinant N15 TCR derivatives

| Expression level (mg/liter) | BaculoN15 | N15 | N15H | iN15Δa | iN15ΔH | N15b | N15Δb | N15ΔA | N15ΔH |
|-----------------------------|-----------|-----|------|--------|--------|------|------|-------|-------|
| (g/liter)                   | 0.3–1     | 3–5 | 5    | 5      | 5      | 5    | 5    | 5     | 5     |

a) Glycosidase sensitivity
b) aβ molecular mass (kDa)

| Glycosidase sensitivity | Resistant | Sensitive | Sensitive |
|-------------------------|-----------|-----------|-----------|
| aβ molecular mass (kDa) | 80–90     | 80–90     | 60–70     |
| β molecular mass (kDa)  | ~43, ~45  | 41, 43, 45| 39        |
| pH (pH)                 | ND        | 6.5–8     | 6.5–8     |
| Crystallization alone   | No        | No        | No        |
| Crystallized with Fab   | Yes       | Yes       | Yes       |

| Molecular mass (kDa) | 80–90 | 80–90 | 60–70 | 50–70 | 40–60 |
|---------------------|-------|-------|-------|-------|-------|
| Glycosidase Refers  | Endo-H |       |       |       |       |
|                     |        |       |       |       |       |
| α subunit chain      | 34     | 36    | 34    | 34    | 34    |
| β subunit chain      | 31     | 34    | 28    | 38    | 32    |
| Crystallization      |        |       |       |       |       |
|                     | No     | No    | No    | No    | No    |
|                      | Yes    | Yes   | Yes   | Yes   | Yes   |
|                      | ~2.8 Å |       |       |       |       |

a) DL, disulfide-linked; NDL, non-disulfide-linked; ND, not determined.
b) Glycosidase refers to endo-H.

* These crystals were too small and clustered to be useful for structural determination.
TCR αβ Heterodimer Crystals

10. Fields, B. A., Ober, B., Malchiodi, E. L., Lebedeva, M. I., Braden, B. C., Ys ern, X., Kim, J.-K., Shao, X., Ward, E. S., and Mariuzza, R. A. (1995) Science 270, 1821–1824
11. Roth, M. (1989) Nature 338, 383–384
12. Samelson, L. E., and Klausner, R. D. (1992) J. Biol. Chem. 267, 24913–24916
13. Wülfing, C., and Plückthun, A. (1995) Immunologist 3, 59–66
14. Novotny, J., Ganju, R. K., Smiley, S. T., Hussey, R. E., Luther, M. A., Recny, M. A., Sileciane, R. F., and Reinherz, E. L. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 8646–8650
15. Ward, E. S. (1992) J. Mol. Biol. 224, 885–890
16. Soo Hoe, W., Lacy, M. J., Denzin, L. K., Voss, E. W., Jr., Hardman, K. D., and Kranz, D. M. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 4759–4763
17. Gregoire, C., Rebai, N., Schweig- guth, F., Necker, A., Mazza, G., Auphan, N., Millward, A., Schmitt-Verhulst, A. M., and Malissen, B. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 8077–8081
18. Weber, S., Traunecker, A., Oliveri, F., Gerhard, W., and Karjalainen, K. (1992) Nature 356, 793–797
19. Lin, A. Y., Devaux, B., Green, A., Sagerström, C., Elliott, J. F., and Davis, M. M. (1990) Science 249, 677–679
20. Slanetz, A. E., and Bothwell, A. L. M. (1991) Eur. J. Immunol. 21, 179–183
21. Engel, M., Ottenhoff, T. H., and Klausner, R. D. (1992) Science 256, 1318–1321
22. Chang, H.-C., Bas, Z. Z., Yao, Y., Tse, A. G. D., Goyarts, E. C., Madsen, M., Kawasaki, E., Brauer, P. P., Sacchettini, J. C., Nathenson, S. G., and Reinherz, E. L. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 11408–11412
23. O'Shea, E. K., Lumb, K. J., and Kim, P. S. (1993) Curr. Biol. 3, 658–667
24. Stanley, P. (1989) Mol. Cell. Biol. 9, 377–383
25. Bebbington, C. R. (1991) Methods 2, 136–145
26. Gill, S. C., and von Hippel, P. H. (1989) Anal. Chem. 182, 319–326
27. Laemmli, U. K. (1970) Nature 227, 680–685
28. Linsley, K., Chan, S.-Y., Chan, S., Reinhold, B., and Reinhold, V. N. (1994) Anal. Biochem. 219, 207–217
29. Maley, F., Trimble, R. B., Tarentino, A. L., and Plummer, T. H., Jr. (1989) Anal. Biochem. 180, 195–204
30. McPherson, A. (1982) in Preparation and Analysis of Protein Crystals (Glick, D., ed) pp. 82–159, Waverly, Baltimore, MD
31. Jancarik, J., and Kim, S. H. (1991) J. Appl. Crystallogr. 24, 409–411
32. Williams, P. J., Wormald, M. R., Dwek, R. A., Rademacher, T. W., Parker, G. F., and Roberts, A. R. (1991) Biochim. Biophys. Acta 1075, 1456–1503
33. Kuroda, K., Geyer, H., Geyer, R., Doerfler, W., and Klent, H.-D. (1990) Virol- ogy 174, 418–428
34. Parekh, R. B., Tse, A. G. D., Dwek, R. A., Williams, A. F., and Rademacher, T. W. (1987) EMBO J. 6, 1233–1244
35. Kube, R. T., Born, W., Kapperl, J. W., Marrack, P., and Pigeon, M. (1989) J. Immunol. 142, 2736–2742
36. Bill, J., Kanagawa, O., Linten, J., Utsonomiya, Y., and Palmer, E. (1990) J. Mol. Cell. Immunol. 4, 269–279
37. Kabat, E., Perry, H., Gottesman, K., and Foeller, C. (1991) Sequences of Proteins of Immunological Interest, 5th Ed., National Institutes of Health, Bethesda, MD
38. Kovari, L. C., Momany, C., and Rossmann, M. G. (1995) Structure 3, 1291–1293, and references therein