Constitutively oxidized CxxC motifs within the CD3 heterodimeric ectodomains of the T cell receptor complex enforce the conformation of juxtaposed segments.

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Running title: CxxC motif intramolecular disulfide bond in CD3γ, δ, and ε

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CAPSULE

Background: CD3 subunits are essential signaling components of the TCR.

Results: The membrane proximal CD3 CxxC motif is constitutively oxidized and critical for subunit conformation.

Conclusion: The CxxC intramolecular disulfide bond is an important structural feature of the CD3 subunits that couples extracellular activating events to intracellular signaling regulation.

Significance: Redox characterization provides insight into CD3 rigidifying elements in mechanotransduction.

ABSTRACT

The CD3εγ and CD3εδ heterodimers along with the CD3ζζ homodimer are the signaling components of the T cell receptor (TCR). These invariant dimers are non-covalently associated on the T cell plasma membrane with a clone-specific (i.e. clonotypic) αβ heterodimer that binds its cognate ligand, a complex between a particular antigenic peptide and an MHC molecule (pMHC). These four TCR dimers exist in a 1:1:1:1 stoichiometry. At the junction between the extracellular and transmembrane (TM) domains of each mammalian CD3ε, CD3γ and CD3δ subunit is a highly conserved CxxC motif previously found to be important for thymocyte and T cell activation. The redox state of each CxxC motif is presently unknown. Here we show using LC-MS and a biotin switch assay that these CxxC segments are constitutively oxidized on resting and activated T cells, consistent with their measured reduction potential. NMR chemical shift perturbation experiments comparing a native oxidized CD3δ CxxC-containing segment with that of a mutant SxxS-containing CD3δ segment in LPPG micelles show extensive chemical shift differences in residues within the membrane-proximal motif as well as throughout the TM and cytoplasmic domains, as a result of the elimination of the native disulfide. Likewise, direct comparison of the native CD3δ segment in oxidizing and reducing conditions reveals numerous spectral differences. The oxidized CxxC maintains the structure within the membrane-proximal stalk region as well as that of its contiguous transmembrane and cytoplasmic domain, inclusive of the ITAM signaling motif. These results suggest that preservation of the CD3 CxxC oxidized state may be essential for TCR mechanotransduction.
INTRODUCTION

The CD3ε, CD3γ, and CD3δ integral transmembrane proteins are components of both the pre-TCR and the TCR complexes and, as such, are required for proper T cell development and cognate recognition of antigen by mature T cells (1-5). The critical role of CD3 has been highlighted in cases of human CD3 deficiency (6-9). Each CD3 subunit contains extracellular, transmembrane (TM) and intracellular components that are necessary to mediate TCR function (10-12). These CD3 subunits interact to form CD3εγ and CD3εδ heterodimers that non-covalently associate with the TCRα and β subunits on the surface of αβ T cells [reviewed in (3)]. After assembly of these three heterodimeric components on the T cell membrane, the CD3ζζ homodimer associates, thereby forming the TCR complex. The TCRα and β chains engage in a direct interaction with antigen upon recognition of surface displayed peptide-MHC complexes (pMHC) on antigen presenting cells, but themselves lack any significant intracellular signaling motifs, being comprised of approximately 5-6 amino acid long cytoplasmic tails (3,13). Conversely, although the CD3 subunits themselves do not participate directly in pMHC antigen recognition, they are critical for relaying TCR-pMHC binding events intracellularly using their ITAM-containing, relatively lengthy (50-60 aa long) cytoplasmic tails (11,14,15). The TM domain of each CD3 subunit is important for TCR complex assembly and is pivotal in linking extracellular binding events to intracellular signaling outcomes (16).

Just bordering the TM domains of CD3ε, CD3γ, and CD3δ is a highly conserved CxxC motif within this membrane proximal region of each subunit. The CD3 CxxC motif is important for CD3εγ and CD3εδ heterodimer association (17-20). Moreover, mutation of the cysteine residues in the CxxC motif has a significant effect on pre-TCR and αβ TCR development and signaling (19,21). While the physiological state of the CxxC motif has not been definitively characterized, it is known that the cysteines residues neither participate in intermolecular disulfide bonding interactions nor in metal ion coordination (19,20). It has been postulated that the adjacent, closely spaced cysteines might form an intramolecular disulfide bond to provide structural stability to the membrane proximal region and TM domain (18). In this regard, CxxC motifs at the N-terminal of α-helices have been observed to form intramolecular disulfide bonds that cap the helix and confer marked stability to the helical structure (22). If this were the case, then the contribution of a CD3 CxxC intramolecular disulfide bond at the N-terminus of the TM domain could provide needed rigidity to the protein subunit to facilitate the recently described mechanosensing properties of the TCR (23-27). The force generated from TCR-pMHC binding interactions may be transmitted to the structurally stabilized CxxC motif in the membrane proximal region and capped TM segment for efficient transfer across the plasma membrane to initiate intracellular signaling cascades in response to extracellular antigen ligation under load [i.e. low piconewton (pN) force].

To investigate these possibilities we employed a combination of NMR, MS, and biochemical techniques to definitively characterize the native redox state of the CD3 CxxC motif on the surface of T cells. We can conclude from our results that each CxxC motif found in the CD3ε, CD3γ, and CD3δ subunits form highly stable intramolecular disulfide bonds. The CxxC disulfide bond remains constitutively oxidized even in the presence of a large excess of chemical reductant or when incubated with thioredoxin. Additionally, the oxidized state is preserved during T cell activation. Loss of the intramolecular disulfide bond either by reduction or mutation induced extensive conformational effects within the CxxC motif that were propagated throughout the TM domain and cytoplasmic tail when examined by NMR measurements using purified CD3 protein segments. The oxidation state of the CxxC is therefore coupled to the conformation of the signaling domain.

EXPERIMENTAL PROCEDURES

Reagents-Trypsin (sequencing grade) was purchased from Promega (Madison, WI), endoproteinase Lys-C (MS grade) from Wako Chemicals USA (Richmond, VA), and endoproteinase Glu-C (sequencing grade from Staphylococcus aureus V8) from Roche applied
science (Indianapolis, IN). PNGase F (proteomics grade), guanidine hydrochloride (Gn-HCl), ammonium bicarbonate, L-dithiothreitol (DTT), iodoacetamide (IAM), N-Ethylmaleimide (NEM), and formic acid (FA, optima LC/MS) were obtained from Sigma-Aldrich (St. Louis, MO). LC-MS grade acetonitrile (ACN) and water was purchased from J.T. Baker (Phillipsburg, NJ, USA).

**His-GB1-CxxC peptides constructs and expression** - The peptide constructs contained an N-terminal His tag, GB1 domain, a Met residue followed by the CxxC motif and 5 residues C-terminal corresponding to each specific mouse CD3 subunit. The poly-ala control peptide sequence used was CAACAAAAKAAAAAKGY (22). Cloned constructs were isotopically expressed in the soluble fraction at yields of approximately 5-10mg/L in M9 minimal media containing 15N ammonium chloride (Cambridge Isotope Labs.) as the sole nitrogen source. Expressed proteins were purified in PBS using cobalt metal affinity resin (Clontech), followed by S75 size exclusion chromatography.

**CD3δ TMC-CxxC and CD3δ TMC-SxxS constructs and expression** - Each construct contained an N-terminal His tag, GB1 domain, a flexible linker containing a TEV protease site followed by the native mouse CD3 sequences beginning at 4 residues N-terminal to the CxxC motif and extending to the C-terminus of the cytoplasmic tail. Cloned constructs were isotopically expressed at yields of approximately 10mg/L in M9 minimal media containing 15N ammonium chloride as the sole nitrogen source. Expressed proteins were solubilized from the insoluble fraction using TBS containing 1% SDS. During solubilization of the CD3δ TMC-CxxC segment, the protein was oxidized with 5 mM GSSG and 2 mM MMTS, whereas CD3δ TMC-SxxS was oxidized with 2 mM MMTS only. The protein segments were purified by cobalt metal affinity resin, and then TEV protease digested in Tris buffer pH 7.5 containing 4 mM dodecyl maltoside (Anatrace) and 4 mM DTT. After complete digestion, the samples were TCA precipitated, washed with acetone, and dried thoroughly. The protein solution by cobalt metal affinity resin and the CD3 segments were purified to homogeneity by size exclusion chromatography. After the final purification step the protein segments were TCA precipitated, washed with acetone, and dried thoroughly. The samples were then dissolved in 30 mM Tris buffer pH 7.0 containing 100 mM LPPG, 10 % D₂O and 0.02 % NaN₃ for NMR analysis. The CD3δ TMC CxxC sample used for completion of the backbone assignments was solubilized in 2H-palmitoyl lysophosphoglycerol (16:0 LPG) buffer (d31-LPPG was obtained from fbreagents.com, Cambridge, MA).

**Jurkat cell culture and reduction experiments** - Rex cells were previously enriched via repeated cell sorting of a high CD3 expressing population from Jurkat cell lines (28). Rex cells were cultured using DMEM media supplemented with 20% heat inactivated fetal bovine serum, L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml) and 5 mM 2-mercaptoethanol. Cells were cultured in a 37°C incubator with 5% CO₂. Rex cells were maintained at a concentration of less than one million cells/ml of media during culture. Over a billion cells were grown from each seed culture and a new stock was thawed for subsequent expansions. Approximately 5 x 10⁸ cells were pooled for each control sample and tested sample condition, washed with 37°C PBS and resuspended in PBS at 37°C for the reduction experiments. For samples investigating CD3 reduction by DTT, the control sample was treated with 6 mM NEM and the reduced sample was treated with 2 mM DTT. Both samples were incubated at 25°C for 10 minutes. Following incubation, 6mM NEM was added to the reducted sample, and then both samples underwent CD3 subunit purification. For samples investigating CD3 reduction by thioredoxin [TRX (Sigma, human recombinant)], the control was treated with 100 µM DTT and the reduced sample was treated with 100 µM DTT and 5 µg/ml of TRX. Both samples were incubated at 25°C for 15 minutes and then 1.5 mM NEM was added to each sample prior to CD3 subunit purification.

**PBMC cell culture and activation**
Leukopacks were obtained from the Jimmy Fund Kraft Family Blood Donor Center at DFCI under an institute approved IRB protocol. PBMC’s were cleaved N-terminal tags were removed from the protein solution by cobalt metal affinity resin and the CD3 segments were purified to homogeneity by size exclusion chromatography. After the final purification step the protein segments were TCA precipitated, washed with acetone, and dried thoroughly. The samples were then dissolved in 30 mM Tris buffer pH 7.0 containing 100 mM LPPG, 10 % D₂O and 0.02 % NaN₃ for NMR analysis. The CD3δ TMC CxxC sample used for completion of the backbone assignments was solubilized in 2H-palmitoyl lysophosphoglycerol (16:0 LPG) buffer (d31-LPPG was obtained from fbreagents.com, Cambridge, MA).

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isolated from leukopacks using ficoll-paque centrifugation and were plated at one million cells/ml using DMEM media supplemented with 20% heat inactivated fetal bovine serum, L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 µg/ml). T cells in PBMC were activated with anti-CD3 antibody [2AD2 (anti-CD3ε) (1:500 dilution) (29) or the combination of mitogenic anti-T112 and anti-T113 anti-CD2 antibodies (1:200 dilution) (30) and 1 µg/ml of PMA]. Antibodies were maintained as frozen ascitic fluid stocks at approximately 1-2 mg/ml as previously described (29,30). The surface of 2Ad2/PMA activated PBMCs were monitored at 0, 1, and 24 hours with anti-CD3 (Clone HIT3a), anti-CD4 (Clone RPA-T4), and anti-CD8 (Clone RPA-T8) in PBS buffer with 5% FBS, 0.1% sodium azide for thirty minutes. Samples were washed, fixed with 4% paraformaldehyde (Sigma) and analyzed using a FACsAria flow cytometer (BD Biosciences, San Diego, CA., USA).

**CD3 purification from Jurkat cells**-Jurkat cells were centrifuged at 2K rpm, and washed twice with ice-cold PBS containing 1.5 mM NEM. The cells were first lysed in 20 mM Tris pH 7.5, 1.5 mM NEM hypotonic buffer, centrifuged at 16K rpm, and the supernatant was removed. The cell pellets were resuspended in TBS lysis buffer containing 1% Triton X-100, 60 mM octyl glucoside, 1.5 mM NEM, 1 mM PMSF, and a Roche protease inhibitor tablet. Lysis proceeded on ice for 15 minutes, and then the samples were centrifuged at 16K rpm. The cell lysate was then added to an anti-CD3ε or concavalin A affinity purification column.

**Anti-CD3ε affinity purification**-Lysate was added to either an anti-CD3ε RW2-8C8 (31) or Leu4 (BD Biosciences) antibody column prepared at a concentration of 5 mg/ml of antibody to protein-G beads, centrifuged at 2000 rpm, and incubated on the column overnight at 4°C with rotation. After incubation, the lysate flowed through the column, and the column was washed extensively with lysis buffer, and the CD3 subunits were eluted with 0.1 M glycine pH 3.0 and neutralized immediately upon elution with Tris buffer. Column fractions were run on non-reducing SDS PAGE gels and silver stained. Those fractions containing bands in the 25 kDa to 15 kDa region, corresponding to the molecular weights of the CD3 subunits, were pooled and concentrated to 0.2 ml using 10 kDa Millipore centrifugal filter unit. Samples of the concentrated eluate were run on non-reducing SDS PAGE gels and Coomassie stained. Bands in the 15 kDa to 25 kDa region were excised and underwent MS analysis.

**Concavalin A affinity purification**-Cell lysate was added to a 2 ml concavalin A column at approximately 10 mg of ConA/ml of bead, (GE Healthcare) and incubated overnight on the column at 4°C with rotation. After incubation, the lysate flowed through the column, which was then washed with RIPA buffer containing 1.5 mM NEM, followed by TBS pH 7.0 containing 1% Triton X-100, 1.5 mM NEM. CD3 was then eluted from the column with TBS pH 7.0 containing 1% Triton X-100, 0.5 M methylmannoside, and 1.5 mM NEM. (CD3 samples treated with TRX or DTT post-lysis and purification lacked NEM from the second column wash step and elution). Samples from the biotin switch assay were directly run on a non-reducing SDS PAGE gel, transferred to PVDF membrane and western blotted for anti-CD3ε (32) or streptavidin-HRP (Pierce) reactivity. Samples undergoing MS analysis were TCA precipitated, washed with acetone, and dried. The samples were then resolubilized in SDS-PAGE gel running buffer containing NEM and separated by non-reducing SDS PAGE. The gel was coomassie stained and bands in the 15 kDa to 25 kDa region were excised and underwent MS analysis.

**Native in-gel digestion**-The gel bands were cut into 1 mm × 1 mm pieces, destained by ACN, and dried in a Speedvac (Labconco, Centrivap Cold Trap). The gel pieces were then rehydrated into the enzyme buffer which was 12.5 ng/µL of enzyme (Lys-C, Trypsin, Glu-C, and PNGase F) in 25 mM ammonium bicarbonate at 4°C for 30 min. Subsequently, the enzyme buffer was replaced by 25 mM ammonium bicarbonate and incubated at 37°C overnight. Finally, the digest was extracted by ACN and concentrated to almost complete dryness in the Speedvac.

**LC-MS**-An Ultimate 3000 nano-LC pump (Dionex, Mountain View, CA) and a self-packed C18 column (Magic C18, 200 Å pore and 5 µm particle size, 75 µm i.d. × 15 cm) (Michrom Bioresources, Auburn, CA) was coupled online to an LTQ-Orbitrap-ETD XL mass spectrometer.
(Thermo Fisher Scientific, San Jose, CA) through a nanospray ion source (New Objective, Woburn, MA). Mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile) were used for the gradient which consisted of (i) 20 min at 2% B for sample loading 0.2 µL/min; (ii) linear from 2 to 5% B for 2 min; (iii) linear from 5 to 35% B for 60 min; (iv) linear from 35 to 90% B for 3 min; and finally (v) isocratic at 90% B for 5 min. The LTQ-Orbitrap-ETD XL mass spectrometer was operated initially in the data-dependent mode as follows: survey full-scan MS spectra (m/z 350-2000) were acquired in the Orbitrap with a mass resolution of 30,000 at m/z 400 (with an ion target value of 5 × 10⁵ ions), followed by nine sequential MS² scans using the LTQ.

Data analysis-The raw data were initially searched against human CD3ε, γ, and δ sequence database in Proteome Discoverer 1.3 with a mass tolerance of ±10 ppm for the precursor ions and ±0.8 Da of product ions with unspecific enzymatic digestion. The final confirmation of the peptide assignment was obtained by manual inspection to match the high abundant product ions with the precursor ion mass accuracy <5 ppm. The intrachain disulfide was assigned by the loss of a hydrogen (-1 Da) at each disulfide-involved cysteine.

CD3 reduction experiments following cell lysis and CD3 purification-Concavalin A purified samples were divided into 200 µl aliquots and 200 µM DTT was added to the control, TRX, and lipoic acid sample. TRX was added at a concentration of 10 µg/ml and lipoic acid (Sigma) at 10 µM to each respective sample. The DTT reduction sample was treated with 1 mM DTT. All samples were incubated at 25°C for 30 minutes and then 1 mM NEM-biotin (Pierce) was added to each sample. The samples were separated by SDS-PAGE, transferred to PVDF membrane and western blotted for anti-CD3ε using a rabbit anti-CD3ε heteroantiser (32) or streptavidin-HRP (Pierce) reactivity.

NMR spectroscopy-NMR spectra were acquired on Bruker 500 and 750, or Varian 700 MHz spectrometers equipped with a 5-mm cryogenic probe. ¹H-¹⁵N heteronuclear single quantum correlation experiments (HSQC) NMR experiments for the His-GB1-CxxC peptides were performed at 298 K. Samples were in PBS buffer containing 2mM GSSG and increasing amounts of GSH were titrated into the sample and a spectrum was recorded at each titration point. Transverse relaxation optimization spectroscopy (TROSY)-enhanced ¹H-¹⁵N HSQC spectra were collected on the CD3δ TMC-CxxC and CD3δ TMC-SxxS protein segments at 310 K. The standard array of 3D TROSY-enhanced triple resonance backbone experiments were carried out on the CD3δ TMC-CxxC segment for completion of the backbone resonance assignments (18). The two indirect dimensions were Non-Uniformly Sampled (NUS), with 12-15% of the 2D grid acquired using a Poisson Gap Sampling. The NUS data were reconstructed using istHMS software (33,34). Linearly acquired data were processed with NMRPipe (35) and all the data was analyzed with NMRView (36) and CARA (37).

RESULTS

The measured equilibrium reduction potentials are consistent with the CD3 CxxC motifs being oxidized on the cell surface. The ectodomain architecture of CD3γδ and relative position of the respective CxxC motifs, TM and cytoplasmic segments is shown in Fig 1. The equilibrium reduction potential of shortened segments of the CD3ε, CD3γ, and CD3δ subunits were determined to elucidate the physiologically relevant state of the CxxC motif on the surface of T cells. The CD3 CxxC-containing segments began with an N-terminal Met residue followed by the CxxC motif and extended five residues C-terminally, and were produced recombinantly as fusions with GB1 protein. Following expression and purification, the standard reduction potentials (E°) were calculated by analysis of changes in HSQC crosspeak intensities (Fig. 2A), reflective of the amounts of reduced and oxidized CxxC peptide, in response to changes in GSH and GSSG composition of redox buffer (38). All CD3 equilibrium reduction potentials were found to be significantly more negative (E° for CD3ε, γ, and δ are -0.211V, -0.221V, and -0.226V respectively, Fig. 2B) than the redox potential of the ER (E°= -0.137 to -0.185V) (39) or extracellular pools of cysteine/cystine, the predominant thiol pair in human plasma (-0.080V) (40). These results substantiate the hypothesis that the CxxC motifs are oxidized during folding in the ER, and are therefore oxidized on the T cell surface. Previous
data probing cell surface CD3γ using anti-CD3γ-Ab specific for its N-terminus showed reactivity with WT CD3γ on the surface TCR complex but not of a SxxS motif variant of CD3γ in which both cysteines of CxxC were mutated to serine (19). Collectively, these data suggest that the normal, active state of CD3-CxxC in CD3γ, CD3δ, and CD3ε on the cellular surface is oxidized and that this state supports proper domain topology.

The CD3 CxxC motif forms an intramolecular disulfide bond on the surface of T cells. To experimentally determine the oxidation status of the CD3 CxxC motif on the surface of resting and activated T cells directly, mAb affinity column purified CD3 was examined by LC/MS analysis. Approximately 0.5 x 10⁹ cells Jurkat REX T cells were harvested and lysed. Initially, the CD3 subunits were isolated from the cell lysate via the anti-CD3ε antibodies, Leu4 or RW2-8C8 (31). The results with each anti-CD3ε mAb were indistinguishable (data not shown). The Fig 3A inset shows the region of the Coomassie stained SDS PAGE from which the CD3 components were excised. As described in the Experimental Procedures, after multi-protease enzymatic digestions followed by LC/MS analysis, the relevant CxxC motif of the CD3γ, CD3δ, and CD3ε subunits was identified (Fig 3A-C). The CxxC peptides were each observed to be oxidized, having a mass 2 daltons lower than the calculated mass for the reduced form, confirming that the CD3 CxxC motifs are in the oxidized state, and forming an intramolecular disulfide bond (Fig. 3), irrespective of the anti-CD3 mAb used for the isolation. The results with CD3ε (Fig 3A) are an exemplar, where the left trace shows the extracted ion chromatogram (XIC) of the m/z (1+) peptide and the insert shows the experimental monoisotopic m/z 825.26 (1+) containing an intrachain disulfide from the mass spectrum. The MS² spectrum of the collision induced dissociation (CID) to the right of the mass spectrum confirms the identity of this peptide.

To remove the potential of antibody bias towards the selective purification of oxidized CD3 subunits, CD3 was also captured from cell lysates with a concanavalin A (ConA) coupled resin. This lectin binds α-mannosyl-α-glucosyl residues and demonstrates high affinity for oligomannose-type N glycans and hybrid type N glycans (41). As such, ConA affinity chromatography can be used to capture the CD3γ and CD3δ subunits that contain such N-linked glycans and the tightly non-covalently associated non-glycosylated CD3ε subunit. LC/MS analysis of ConA purified CD3 subunits yielded comparable results to those shown in Fig. 3 and as summarized in Table 1. We conclude that the resting, native state of the CD3 CxxC motif of CD3γ, CD3δ, and CD3ε on the T cell surface is that of an intramolecular disulfide, regardless of the isolation method.

The CD3 CxxC motif is highly resistant to reduction. Next, the potential for modulation of the CxxC oxidation state on T cells was also explored. Initially, Jurkat T cells were incubated with either 2 mM or 10 mM DTT prior to lysis in the presence of N-ethylmaleimide (NEM), isolation of CD3, and LC/MS analysis. However, in the presence of either concentration of DTT, the CxxC motif remained oxidized (Table 1). In fact, reduced CxxC peptides could only be detected by LC/MS after isolation from the cell surface into a detergent solution and subsequent treatment with DTT and NEM just prior to SDS PAGE gel separation (Table 1). These results suggest that the CxxC motif may be occluded on the T cell surface, with reductant unable to access this region until CD3 is extracted from the cell membrane.

Biotin Switch Assay. In addition to MS analysis, reduction of CD3 by DTT was also monitored using a biotin-switch assay [reviewed in (42)]. In this assay, reduced cysteine residues are modified by the addition of NEM-biotin and subsequently detected by streptavidin-HRP chemiluminescence after separation via SDS-PAGE and transfer to PVDF membrane. 

While a notable increase in reduced proteins was observed in the DTT treated samples in the 60-200kDa range (Fig. 4A, left panel lanes), CD3 did not give rise to observable streptavidin-HRP signal and hence remained oxidized, consistent with the MS results (Fig. 3). Note that anti-CD3ε rabbit antisera Western blotting readily detected CD3 in the samples. The minor CD3ε western blot signal observed at approximately 40 kDa represents CD3ε-CD3ε misfolded intracellular proteins (39), further enhanced through rapid dimer formation after DTT treatment.

Thioredoxin is incapable of reducing the CxxC disulfide bond. Although the small
molecule reductant DTT did not reduce the CxxC motif on the cell surface, the ability of the CD3 subunits to undergo enzyme mediated reduction was then tested by the addition of exogenous thioredoxin (Trx) to Jurkat T cells. Trx is known to be secreted into the extracellular space and found to regulate cellular functions (43-45). The cells were incubated with varying amounts of Trx and then lysed and purified as described above. MS analysis of Trx treated cells showed that the CxxC motif remained oxidized under the conditions tested (Table 1). The ability of Trx to reduce the CD3 subunits after isolation from the cell membrane, where they are potentially more accessible to reduction, was analyzed by using the gel-based biotin switch assay. CD3 was purified from the cell lysates via ConA resin, then Trx was added with 10 \( \mu \text{M} \) DTT to undergo enzymatic mediated reduction of the CxxC motif on the cell surface, the ability of the CD3 molecule reductant DTT did not reduce the CxxC motif of the CD3 surface expression on mature \( \varepsilon \)-CD3 pool lost more slowly. Figure 5B shows that the CD3 surface expression on mature CD8 T cells by FACs analysis is rapidly lost upon 2Ad2 addition, going from a mean fluorescence intensity (MFI) 4000 to 200 in just 1 hour. Although not shown, a similar pattern of CD3 loss was detected on CD4 T cells. Unlike CD3, other proteins co-purified by the ConA column do display an increase in reduction over the course of activation by this assay (Fig 5A, right panel). This phenomenon has been previously reported in the literature (49).

Next, to bypass the CD3 pathway and the concomitant TCR down modulation, T cells were activated via the CD2 pathway using the combination of mitogenic anti-T112 and anti-T113 antibodies (30). Cell samples were removed after 6 days when cellular proliferation was already maximal (30) and treated with biotin-NEM. With this stimulation, the levels of CD3 remain constant over the activation period [(30) and data not shown].

The CD3 oxidation state is unaffected by T cell activation via the TCR complex or CD2 pathways. To determine if the oxidation state of CD3 could be modified by activation, T cells isolated from PBMCs were characterized in the resting and activated state by monitoring disulfide bond reduction using the biotin switch assay. T cells were activated via the CD3 \( \varepsilon \) pathway using the anti-human CD3 2Ad2 murine IgM isotype antibody (29) for various times. Cell samples were removed after 0, 1, 24, and 48 hours, and treated with biotin-NEM. A decrease in the amount of CD3 was observed over the course of the activation (Fig 5A, left panel). This result is expected, since activation via the TCR complex either with anti-CD3 mAb plus PMA or antigen results in loss of CD3 expression both by internalization and membrane shedding (29). The detectable CD3 remained oxidized in the resting and activated cells as tracked through streptavidin-HRP chemiluminescence (Fig 5A, right panel). The CD3 \( \varepsilon \) dimer, as observed in Fig. 4A and described previously (48), decreases at a slower rate. Perhaps this dimer represents an internal CD3\( \varepsilon \)-CD3\( \varepsilon \) pool lost more slowly. Figure 5B shows that the CD3 surface expression on mature CD8 T cells by FACs analysis is rapidly lost upon 2Ad2 addition, going from a mean fluorescence intensity (MFI) 4000 to 200 in just 1 hour. Although not shown, a similar pattern of CD3 loss was detected on CD4 T cells. Unlike CD3, other proteins co-purified by the ConA column do display an increase in reduction over the course of activation by this assay (Fig 5A, right panel). This phenomenon has been previously reported in the literature (49).
shown]. Nonetheless, biotin-modified CD3 (i.e. reduced CD3) was not detectable in the resting and activated T cells despite an overall observable increase in multiple reduced proteins that are ConA co-purified from the cell samples (Fig 5). The combined cellular results strongly support the notion that the CD3 CxxC motif is stably oxidized in both resting and activated T cells; furthermore, the disulfide bond is resistant to reduction via DTT, Trx or T cell activation.

A structural role for the CxxC motif in maintenance of TM and cytoplasmic segment conformations as revealed by TROSY $^1$H-$^{15}$N HSQC spectra. The structural role of the CxxC motif was then investigated using two approaches. In the first approach, a double Cys to Ser mutant of the CD3δ segment (SxxS) was prepared and compared to the wildtype CD3δ segment (CxxC) by NMR spectroscopy. Each construct began 4 residues N-terminal to the CxxC motif and extended to the C-terminal end of the cytoplasmic domain (Fig. 1). The protein segments were $^{15}$N isotopically labeled during recombinant expression, purified to homogeneity, and then incorporated into LPPG micelles for NMR analysis. The chemical shift dispersion in the TROSY-$^1$H-$^{15}$N HSQC spectrum of the CxxC construct was consistent with that of a membrane spanning motif coupled with a partially structured soluble cytoplasmic tail (data not shown); Resonances were spread within a relatively narrow range characteristic of either alpha helical or random coil secondary structure. Resonances associated with transmembrane residues (Resonances 80-102) were uniformly less intense than those of the more mobile cytoplasmic region residues (Resonances 103-151). Upon comparison of the TROSY-$^1$H-$^{15}$N HSQC spectra of the CxxC and SxxS segments, extensive chemical shift changes were readily observed in residues C71, T81, and I91. Based on the partial backbone assignments, the largest chemical shift changes occurred in the N-terminal portion of the protein segment that includes the membrane-proximal region where the native CxxC motif resides (Fig. 6, resonances 67-78). Of significance, the chemical shift changes also extend C-terminally into the TM and cytoplasmic domains. Characterization of the spectral differences between the CxxC and SxxS protein segments suggests that the membrane proximal CxxC plays an important structural role in governing the conformation of the CD3 segment that includes the intracellular signaling domain (resonances 103-151).

In the second approach, chemical reduction of the native CxxC motif in the CD3 segment was also investigated by NMR spectroscopy. A titration experiment from 0 mM to 8 mM DTT was carried out using the wildtype CD3δ segment described above. Consistent with the CxxC vs. SxxS chemical shift comparison, considerable spectral differences were observed in CD3δ upon addition of DTT, most notably in the N-terminal CxxC region (Fig. 7). As shown in Fig 7A, comparison of 0 mM and 8 mM DTT, specific identified changes include C71, N73, and D78. Chemical shift changes were also detected in the TM and cytoplasmic domains T81, L95, A119. Interestingly, Fig. 7B, which focuses on one region of the spectra with additional DTT amounts and incubation kinetics shown, indicates that the CxxC disulfide bond appears occluded, as was observed in the T cell experiments. Thus complete reduction of the protein segment was not observed until after a significant lapse of time (i.e. 12 hours) even in the presence of 4-fold molar excess DTT (4mM). Taken together, these results suggest that the highly favored, intramolecular disulfide bond in the CD3 CxxC motif appears protected from reduction in a membrane and membrane-like environment.

DISCUSSION

The CD3ε, CD3γ, and CD3δ membrane proximal CxxC motif is an important and highly conserved structural element within each of the CD3 subunits of all mammalian species. Based on the presented results, the CxxC intramolecular disulfide bond is likely formed during the folding process in the endoplasmic reticulum and this oxidized state is then preserved on the surface of T cells. Loss of the disulfide bond either by mutation or reduction has a significant impact on the conformation of the protein segment in the vicinity of the CxxC motif, and also impacts the positioning of the TM domain and cytoplasmic tail as shown for CD3δ. It is expected that CD3γ and CD3ε segments behave in a similar manner to CD3δ as evidenced by the similarity of their reduction potentials and behavior on the surface of T cells. The results herein thus bolster a model in
which the CD3 CxxC motifs rigidify the structure of CD3 heterodimeric subunits within the extracellular to intracellular junction, potentially mechanically coupling the position and motion of the extracellular TCR complex to the intracellular signaling motifs. This rigidification adds to that of the squat ectodomains paired through extensive interface contacts and conjoint G strands (50).

Mutational studies of the cys residues in the CD3γ and CD3ε CxxC motif have been shown to have significant physiological effects. For example, mutation of CD3γ cys residues leads to structural alterations in both the CD3γ and CD3ε subunit when measured by conformationally specific antibody binding, and also resulted in impairment of CD3εγ heterodimeric pairing (19). Cellular studies completed on CD3ε CxxC cys mutants recently disclosed defects in TCR-dependent development and activation. These irregularities are presumably due to dysfunction induced by the CD3ε mutation in both CD3εγ and CD3εδ heterodimers (21). However, CD3γ mutation alone caused dysfunction of the pre-TCR as judged by attenuated DN3 and DN4 thymocyte transition and impairment in developmental progress to the DP stage without reduction in surface receptor expression. (19). The CD3 CxxC cys mutant subunits also appeared to affect TCR complex intersubunit interactions (51,52), with observed diminished membrane proximal mediated association of CD3εδ with TCRα in an in vitro translation experimental system (20). While there appears to be some variability in the impact of the cys mutations on subunit association and T cell functioning ascribed to each CD3 subunit, preservation of the CxxC motif is undoubtedly critical for proper TCR subunit positioning and cellular signaling.

The standard reduction potentials of -0.226 V, -0.211 V, and -0.221 V for CD3δ, CD3ε, and CD3γ CxxC motifs respectively are between the calculated values of the CxxC disulfide bonds found in PDI (-0.175 V) and thioredoxin (-0.270 V) (53). The CD3ε value is observed to be slightly less negative than CD3δ and CD3γ, which may be due to the presence of the more bulky aromatic side-chain from a tyr residue in the intervening sequence (Fig. 1). The reduction potentials for all three CD3 segments are considerably more negative than the redox potential of the ER, where redox-mediated folding occurs (39). The calculated values agree with the experimental data showing only oxidized CD3 CxxC motifs on the cell surface.

Interestingly, we observed herein an inability to reduce the CD3 CxxC motifs on the T cell surface after treatment with the chemical reductant, DTT, or the enzyme-mediated reductase, Trx. In contrast, in the soluble, non-membrane associated peptide model system it was possible to reduce the disulfide bond with increasing amounts of reductant. On the other hand, a significant number of cell surface proteins did become more reduced after treatment with DTT or Trx in the intact cellular experiments, despite CD3ε, CD3δ and CD3γ remaining oxidized. A similar phenomenon was observed during the T cell activation experiments. The CD3 subunits remained oxidized whether the T cell was activated through the TCR or CD2 pathways; however, an overall increase in other reduced, co-purifying proteins was detected. Collectively, these data suggest that the CxxC region is not accessible to reduction in the TCR complex. Thus, while the peptide model system is optimal for determining the thermodynamic properties of the disulfide bond, it is problematic when considering the CD3 subunits embedded in a lipid environment. Given that CD3 CxxC disulfides were resistant to reduction by the high concentrations of DTT present in the cellular experiments, beyond which cellular viability was compromised, it is unlikely that an environment exists in which these motifs are reduced at the surface of living cells in vivo. Hence, we conclude that CD3 CxxC motifs are not redox switches linked to T cell activation.

The completeness of the oxidized status and the difficulty in reducing the CxxC motifs in a membrane-like environment may be a result of one or more factors. Firstly, there may be a contribution to the stability of the disulfide through thermodynamic coupling to the formation of the transmembrane helix as has been reported in model studies on soluble helices (22,54). Indeed, the reduction potentials of the CD3 motifs may be significantly more negative than those determined here, as is the value for the polyA helix determined by Iqbalysyah et al compared to that shown here (-230mV versus -218mV). Secondly, there may be some occlusion of the site due to its
location within the CD3 molecule itself and/or due to ectodomain quaternary associations within the TCR complex (50). While this is not a factor in experiments studying the isolated CD3δ fragment, the position of the CxxC motif proximal to the central G-strands of the CD3 heterodimers means that it is likely that significant steric hindrance would be encountered by an attacking thiolate anion, which must approach in line with the existing disulfide bond in order for thiol-disulfide exchange to occur. Similarly, the CxxC motif may be partially occluded by the lipid bilayer itself, with the transmembrane helix retaining sufficient rigidity to prevent access by an incoming nucleophile to the disulfide bond.

In conjunction with cellular defects, substantial structural alterations are directly observed by NMR with mutation of the Cys to Ser residues in the CD3δ CxxC motif-containing protein segment. The extensive chemical shift changes observed in CD3δ-CxxC relative to CD3δ-SxxS that occur throughout the protein segment are consistent with the broad structural effects of CxxC mutation impacting conformationally specific antibody detection observed previously with CD3γ (19). Moreover, the large structural changes are suggestive of the importance of the CxxC intramolecular disulfide bond on maintaining the structural rigidity of the membrane proximal region and TM domain, which also appears to be coupled to the positioning of the cytoplasmic tail. One may then infer that the tightly paired and associated G-strands N-terminal to the membrane proximal region in the CD3 extracellular heterodimer (Fig. 1) will be similarly structurally perturbed with loss of the CxxC disulfide bond, thereby linking mechanosensor function of the αβ TCR to the redox state of the CD3 subunits as described below.

Additionally, loss of the intramolecular CxxC disulfide bond through reduction by DTT results in significant conformational changes in the protein segment as was observed with mutation. It is noteworthy that the ability to reduce the CD3δ CxxC disulfide bond in a membrane-like environment in the presence of a large molar excess of DTT was not immediate (Fig 7). This is in agreement with the inability to reduce the CxxC motif in the CD3 subunits displayed on the surface of T cells, and provides further evidence that the CxxC is not readily accessible for reduction in a lipid environment. Therefore, given the likely close proximity of the CxxC motif to the plasma membrane in the native state, mutation or reduction would then affect the disposition of the CD3 segments on the plasma membrane consistent with data on anti-CD3γ ectodomain antibody binding (19). Significant cell surface positional changes would most certainly correlate to conformational changes throughout the TM domain and into the cytoplasmic tail, therefore in agreement with the observed conformational changes in the CD3δ segment induced through mutation or reduction monitored by NMR.

CxxC motifs present at the N-terminus of helical peptides have a propensity to form a helical cap and contribute to a measurable increase in helical stability (22,54). Thus, the intramolecular disulfide bond formed by the CD3 CxxC motif may function to stabilize the TM domain helix and further rigidify the membrane proximal region either by formation of a helical cap or other structural element constrained by the disulfide bond. This rigidity will contribute to the overall CD3 heterodimeric stability within the TCR and contributes to the rigid CD3 heterodimer connectivity to the T cell membrane. The TCR will then become more responsive to force induced pMHC binding events, and thereby better equipped to convert binding energy into intracellular signaling cascade activation (3).

TCR-dependent recognition results from the cross-junctional TCR–pMHC binding at the T cell–APC interface, i.e., a two-dimensional (2D) binding interface, that is undoubtedly subjected to mechanical forces. As noted previously, the TCR complex is composed of an immunoglobulin Fab-like αβ heterodimer and the non-covalently associated CD3 signaling components. The juxtaposition of the squat and rigid heterodimeric CD3 structures on short stalks that flank the taller αβ heterodimer, itself tethered to the T-cell membrane by long linkers, suggested a TCR-based signal transduction mechanism initiated by mechanical triggering of the extracellular domains (21). Dynamic mechanosensing might occur during T-cell immune surveillance, as well as accompanying molecular rearrangement at the immunological synapse post-cessation of cell scanning via outside-in and inside-out force,
respectively. This force-driven signaling notion has recently been confirmed by optical tweezer experiments (25) and extended by biomembrane force probe (BFP) studies demonstrating that force prolongs TCR–pMHC bond lifetime for agonist peptides (55). Given that the TCR functions as a mechanosensor to communicate the pMHC-specific information from the ligand-binding site on the αβ TCR ectodomains through the plasma membrane to the phosphorylation sites of the cytosolic tails of the CD3 subunits via a mechanical process, its architecture is finely tuned to detect, amplify and transduce piconewton and nanometer scale mechanical events through its structural features. In this regard, the uniquely elongated TCRβ constant domain FG loop can push on the CD3εγ ectodomain during mechanical movement as described (4, 21). It now seems clear that the rigidity imparted by the CxxC motifs of the CD3 heterodimers will efficiently facilitate signaling to and through the membrane, on the one hand, and offer a registered vertical alignment on the membrane to optimize signaling in a coordinated fashion, on the other.

An increasing number of receptors appear to work via mechanotransduction, that is the explicit ability of force to induce a biochemical signal [reviewed in (56,57)]. The von Willebrand factor (VWF) protein provides one example of a structure highly reinforced through the presence of disulfide bonds (58,59). VWF contains a number of disulfide bonds that contribute to the global stability of the protein and a C-terminal domain containing a cystine knot motif. The intertwined cys residues respond to a pulling force under sheer stress that then leads to conformational changes in VWF protein binding, ultimately regulating blood coagulation responses (58,59).

In sum, the CD3 CxxC motifs form highly stable intramolecular disulfide bonds on the surface of T cells that appear to be critical in maintaining the conformation of the CD3 subunits, TCR intersubunit interactions, and intracellular signaling responses to extracellular TCR-pMHC binding interactions. It is clear that as our understanding of biology becomes more precise, then the fine structural details underpinning the extraordinary sensitivity and specificity of receptor function will be revealed.
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FIGURE LEGENDS

Fig. 1. Domain architecture of the CD3 subunits. (A) The heterodimeric CD3εγ subunit complex is illustrated, the CD3ε subunit is drawn in blue and the CD3γ subunit in yellow. Select interdomain hydrogen bonds are shown on the G strands, in which the amide protons are drawn in gray and the carbonyl oxygen atoms are drawn in red. The cytoplasmic tails are illustrated vertically to depict receptor length, whereas physiologically, the tails may be associated with the inner leaflet of the plasma membrane. Each CD3 subunit contains an extracellular, transmembrane, and cytoplasmic domain. The CD3εγ extracellular domain structure was generated from the deposited PDB file 1XMW (18) using PYMOL (60). (B) The partial mouse CD3 amino sequences are displayed, initiated at the membrane proximal region in each subunit. The TM domains are underlined. The boxed region drawn on each sequence highlights the CxxC motif. The sequence emphasized with a line above represents the peptides generated for measurement of the equilibrium reduction potentials. The CD3δ Cys to Ser mutations are denoted with red S labels above the native C labels in the sequence.

Fig. 2. Calculated equilibrium reduction potentials of the CD3 CxxC motifs. Peptides containing the CxxC regions of murine CD3ε, γ, and δ, as well as a poly-Ala containing peptide as a control, were produced in E.coli as 15N isotopically labeled 6xHis-GB1 fusion proteins. The peptides included a N-terminal Met residue, the CxxC motif, and five additional residues C-terminal. To aid in sample handling, the protein segments did not include the significantly hydrophobic TM residues. (A). During the titration experiments, the GB1-peptides were equilibrated with 2mM GSSG and varying amounts of GSH (0, 3, 5.1, 9, 15.6, 27, and 46mM) were added and monitored using 1H-15N HSQC spectra. The chemical shift intensities of non-GB1 residues were monitored in a completely oxidizing environment (blue) to a significantly reducing environment (red). (B). Shown are curves fit to data determining the K_eq of each CxxC. Each dot in the curve plots for the titration points represents an individual resonance monitored during the experiment. Standard reduction potentials were calculated from the equilibrium data for CD3γ, δ, and ε and are determined to be -0.221V, -0.226V, and -0.211V respectively. The Poly-Ala control reduction potential was -0.218V, in agreement with the published value of -0.230V of a longer, more helical peptide.

Fig. 3. MS analysis of the oxidation status of the CxxC motif from CD3 subunits isolated from T cells (A) Antibody purified CD3 subunits isolated and concentrated samples from Jurkat T cells separated by SDS PAGE and Coomassie stained. The region excised for MS analysis is illustrated. (A) CD3ε. The identification of disulfide-linked VCENCME by mass spectrometry. The extracted ion chromatogram (XIC) of the peptide is shown from the C18 reversed phase elution with an inset showing the experimental m/z (1+) of the peptide. The experimental monoisotopic m/z 825.26 (1+) ion indicates an intra-chain disulfide (theoretical monoisotopic m/z 827.27, 1+). The right figure shows the CID-MS² spectrum. The fragment ions confirm the identity of peptide VCENCME. (B). CD3γ. The identification of disulfide-linked MCQNCIE by mass spectrometry. The XIC of the peptide is shown from the C18 reversed phase elution with an inset showing the experimental m/z (1+) of the peptide. The experimental monoisotopic m/z 838.29 (1+) ion indicates an intra-chain disulfide (theoretical monoisotopic m/z 840.30, 1+). The right figure shows the CID-MS² spectrum. The fragment ions confirm the identity of peptide MCQNCIE. (C). CD3δ. The identification of disulfide-linked MCQSCVE by mass spectrometry. The XIC of the peptide is shown from the C18 reversed phase elution with an inset showing the experimental m/z (1+) of the peptide. The experimental monoisotopic m/z 797.26 (1+) indicates an intra-chain disulfide (theoretical monoisotopic m/z 799.28, 1+). The right figure shows the CID-MS² spectrum. The fragment ions confirms the identity of peptide MCQSCVE.
Fig. 4. Analysis of the oxidation state in T cells by the biotin switch assay. (A). 0.5 x 10^9 Jurkat REX cells were either untreated (-) or treated (+) with 2 mM DTT followed by biotin-NEM addition. Then CD3 was isolated from the cell lysate with ConA resin and then separated by SDS PAGE gel. Proteins were then transferred to PVDF membrane and blotted for streptavidin-HRP signal or for anti-CD3ε reactivity. The bracketed region from 60 kDa-above 200 kDa represents multiple proteins that have been reduced and biotin modified. The band migrating at approximately 40 kDa represents a small fraction of CD3 dimer. (B). CD3-containing samples were initially ConA purified from untreated Jurkat REX T cells and then samples were either left untreated or treated with: Trx, DTT-RT (Room Temperature), lipoic acid, or DTT and boiling before separation on SDS PAGE gel. Proteins were then transferred to PVDF membrane and blotted for streptavidin-HRP signal or for anti-CD3ε reactivity. The band demarcated with an asterisk (*) represents exogenous Trx that had been added to the sample and became NEM-biotin modified during the sample processing step. The reduced and biotin modified CD3 subunits are highlighted with a white arrow.

Fig. 5. Conservation of the CxxC disulfide bond in both resting and activated T cells. (A) 0.5 x 10^9 T cells isolated from PBMCs were either left untreated or treated with the activating antibody 2Ad2/PMA for 1-48 hours. (B) CD3 staining on the surface of 2Ad2/PMA activated T cells was monitored at 0, 1, and 24 hours with anti-CD3 FITC and anti-CD8 APC. The unstained background control is depicted with the red line and the anti-CD3 FITC staining gated on CD8 APC positive T cells is depicted with the blue line. (C) 0.5 x 10^9 T cells isolated from PBMCs were either left untreated or treated with the activating anti-T112 and anti-T113 antibody combination for 6 days. In both (A) and (C) CD3 was isolated from the cell lysate with Con A resin and then separated by SDS PAGE gel. Proteins were then transferred to PVDF membrane and blotted for streptavidin-HRP signal or for anti-CD3ε reactivity.

Fig. 6. Structural alterations in CD3δ by SxxS mutation. ^1H-15N TROSY HSQC spectrum of CD3δ-CxxC (black) overlaid with the TROSY HSQC spectrum of CD3δ-SxxS (red). Representative resonances are labeled in the membrane proximal, transmembrane and cytoplasmic tail of CD3δ. Numbering corresponds to the full length murine wildtype CD3δ protein where the CxxC motif is at residues 95-98 (Fig. 1 A).

Fig. 7. Structural effects of disulfide bond reduction of the CD3 delta CxxC motif. (A). ^1H-15N TROSY HSQC spectrum of CD3δ-CxxC (red) overlaid with the TROSY HSQC spectrum of CD3δ in the presence of 8 mM DTT (blue). (B). The boxed portion of the TROSY HSQC spectra of CD3δ in panel A is expanded for greater detail of illustrative residues over the course of the DTT titration experiment. Included in this view is a sample where 4 mM DTT was added without (yellow) or with (green) an additional 12 hr incubation prior to NMR analysis. The 0 mM (red) and 8 mM (blue) DTT conditions are also superimposed.

Table 1. Summary of the CD3 redox state results analyzed by LC/MS or via the biotin switch assay under varying experimental conditions.
| CD3 target | Cell type | Treatment | CD3 isolation method | Gel sample additions | Redox status | Analysis method |
|------------|-----------|-----------|----------------------|----------------------|--------------|-----------------|
| Purified Protein | E.coli | (-) | NA | NEM | Oxidized | LC/MS |
| | | (-) | NA | DTT(50 mM)/NEM | Reduced | LC/MS |
| | Jurkat | DTT(200 μM) | NA | NEM | Oxidized | Biotin assay |
| | | DTT(200 μM)/Trx (10 μg/ml) | NA | NEM | Oxidized | Biotin assay |
| | | DTT(200 μM)/Lipoic acid (10 μg/ml) | NA | NEM | Oxidized | Biotin assay |
| | | DTT(1 mM) | NA | NEM | Reduced | Biotin assay |
| | | DTT(1 mM)/Boiling | NA | NEM | Reduced | Biotin assay |
| | Intact Cells | Jurkat | (-) | RW2-8C8 mAb | (-) | Oxidized | LC/MS |
| | | | (-) | RW2-8C8 mAb | NEM | Oxidized | LC/MS |
| | | | (-) | Leu4 mAb | NEM | Oxidized | LC/MS |
| | | | (-) | ConA lectin | NEM | Oxidized | Biotin assay |
| | | | (-) | RW2-8C8 mAb | DTT(50 mM)* | Oxidized | LC/MS |
| | | | (-) | RW2-8C8 mAb | DTT(50 mM)/NEM | Reduced | LC/MS |
| | | | DTT(2 mM) | RW2-8C8 mAb | NEM | Oxidized | LC/MS |
| | | | DTT(2 mM) | Leu4 mAb | NEM | Oxidized | LC/MS |
| | | | DTT(2 mM) | ConA lectin | NEM | Oxidized | LC/MS |
| | | | DTT(2 mM) | ConA lectin | NEM | Oxidized | Biotin assay |
| | | | DTT(10 mM) | ConA lectin | NEM | Oxidized | LC/MS |
| | | | Trx (0.5 μg/ml)/DTT(10 μM) | ConA lectin | NEM | Oxidized | LC/MS |
| | | | Trx (5 μg/ml)/DTT(10 μM) | ConA lectin | NEM | Oxidized | LC/MS |
| | | | 2Ad2/PMA | ConA lectin | NEM | Oxidized | LC/MS |
| | PBMCs | 2Ad2/PMA | ConA lectin | NEM | Oxidized | Biotin assay |
| | | | T11.2/T11.3 | ConA lectin | NEM | Oxidized | Biotin assay |

*a Purified CD3 or intact cells were either not treated (-) or treated with potential reductant (DTT, Trx, lipoic acid, or mAb cellular activation (2Ad2/PMA or T11.2/T11.3)) prior to analysis of the redox status.

*b Despite the addition of DTT, the disulfide bond readily reforms on the SDS PAGE gel without cysteine methylation via NEM
Figure 3
Figure 4

A  Intact cells

Detection:  

|            | Strep-HRP | anti-CD3ε |
|------------|-----------|-----------|
| DTT:       |  -       | -         |

B  Lectin purified CD3

|                | anti-CD3ε |
|----------------|-----------|
| Control        |           |
| Trx            |           |
| DTT-RT         |           |
| Lipase acid    |           |
| DTT-boil       |           |

Strep-HRP

|                |            |
|----------------|------------|
| Control        |            |
| Trx            |            |
| DTT-RT         |            |
| Lipase acid    |            |
| DTT-boil       |            |

* Marker
Figure 5
Constitutively oxidized CxxC motifs within the CD3 heterodimeric ectodomains of the T cell receptor complex enforce the conformation of juxtaposed segments
Kristine N. Brazin, Robert J. Mallis, Chen Li, Derin B. Keskin, Haribabu Arthanari, Yuanwei Gao, Shiaw-Lin Wu, Barry L. Karger, Gerhard Wagner and Ellis L. Reinherz

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Constitutively oxidized CXXC motifs within the CD3 heterodimeric ectodomains of the T cell receptor complex enforce the conformation of juxtaposed segments.

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There were errors in the legends to Figs. 2 and 7 as well as several references as noted below. These changes do not affect the conclusions of this work.

PAGE 18884:
In the legend to Fig. 2, the following sentence should be changed as follows. "Peptides containing the CXXC regions of murine CD3ε, -γ, and -ε as well as..." should read as "Peptides containing the CXXC regions of murine CD3ε, -γ, -δ as well as...">

PAGE 18889:
In the title to Fig. 7, "Structural effects of disulfide bond reduction of the CD3Δa CXXC motif" should instead read as "Structural effects of disulfide bond reduction of the CD3δ CXXC motif."

PAGE 18890:
In the right column, line 18, Ref. 21 should be changed to Ref. 25 instead. "The juxtaposition of the squat and rigid heterodimeric CD3 structures on short stalks that flank the taller αβ heterodimer, itself tethered to the T-cell membrane by long linkers, suggested a TCR-based signal transduction mechanism initiated by mechanical triggering of the extracellular domains (25)."

In the right column, line 37, the citation for Refs. 4 and 21 should be changed to Refs. 3 and 25 instead. "In this regard, the uniquely elongated TCRβ constant domain FG loop can push on the CD3εγ ectodomain during mechanical movement as described (3, 25)."

PAGE 18892:
Ref. 50 should read as shown below.

50. Sun, Z. Y., Kim, S. T., Kim, I. C., Fahmy, A., Reinherz, E. L., and Wagner, G. (2004) Solution structure of the CD3εδ ectodomain and comparison with CD3εγ as a basis for modeling T cell receptor topology and signaling. Proc. Natl. Acad. Sci. U.S.A. 101, 16867–16872

Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.