Introduction

Upon ligand binding, membrane receptors have to transmit information from their ectodomains to their cytoplasmic tails, and several mechanisms have been proposed to account for how this happens. One way would be to simply increase the local concentration of cytoplasmic tails by promoting aggregation of the ectodomains, which can be achieved by crosslinking with a multivalent ligand, or could even be induced by a monovalent ligand, if this binding promotes a conformational change which results in aggregation. The archetypal receptor aggregation mechanism is the activation of membrane receptor tyrosine kinases. Dimerization of these receptors results in activation of their cytoplasmic tyrosine kinase domains and auto-trans-phosphorylation of their tails [1,2]. A second possible mechanism involves a conformational change in the cytoplasmic tails themselves, somehow transmitted from the ectodomains. The clearest example of this is provided by G protein-coupled receptors [3,4]. The aggregation and conformational-change mechanisms are not mutually exclusive; indeed it has been suggested that membrane protein tyrosine kinase receptors also undergo a conformational change [5]. To date, much evidence has been gathered which strongly suggests that several receptors of immunological interest undergo a conformational change upon ligand binding. These include the erythropoietin receptor, the tumor necrosis factor receptors, Fas, the interleukin-6 receptor, and the B cell receptor [6–10]. In multichain membrane receptors the conformational change induced upon ligand binding could be a result of rearrangement of the quaternary structure of the complex.

The T cell receptor (TCR) complex is composed of ligand-binding subunits (TCRα and TCRβ) and signal transducing subunits (CD3ε,CD3γ,CD3δ,CD3ζ and CD3ρ [CD247]) [11–13]. The ligand of the TCR consists of a peptide antigen bound to major histocompatibility complex (MHC) class I or class II molecules. Assembly studies, transfection and reconstitution experiments, and detergent dissociation studies suggest that the TCR complex components are organized as dimers [reviewed in [14]]. CD3ζ forms non-covalently-bound alternate dimers with CD3γ and CD3δ, TCRζ forms disulfide-linked dimers with TCRβ, and CD3ζ is expressed in the form of disulfide-linked homodimers.

In spite of significant advances in understanding how the TCR signal is propagated within the cell, little is known about the mechanisms that initiate TCR signaling. A number of models have been proposed, including oligomerization of the TCR complex [15,16], conformational changes occurring within a single TCRα/β heterodimer or within the complete TCR complex [17,18], geometrical rearrangements within a multivalent TCR complex [19], and segregation of tyrosine kinases and phosphatases from the TCR complex [20,21]. The poor ability of monovalent (Fab) anti-CD3 antibodies to stimulate T cells compared with bivalent antibodies has long suggested ligand-induced oligomerization as a necessary component of the activation mechanism [22–24]. This model has been reinforced by experiments comparing monomeric...
Figure 1. Effect of TCR ligation on the susceptibility of CD3ε and CD3ζ to limited proteolysis. (A) A cartoon of the CD3ε and CD3ζ subunits showing potential trypsin cleavage sites (arrows). Transmembrane domains are indicated with striped boxes, the polyproline sequence (PPS) with an open box, and the foreign Flag epitope, appended to the N-terminus of CD3ε, with a dotted square. The relative positions of the ITAMs within the cytoplasmic tails are marked. (B) Addition of an anti-CD3 antibody that induces TCR conformational change protects the tail of CD3ε from trypsin proteolysis. The indicated concentrations of anti-CD3 (OKT3) or anti-Flag antibody were added to a detergent lysate of Jurkat cells transfected with Flag-CD3ε before incubation with trypsin. The bands corresponding to CD3ε and its degradation products were detected by immunoblotting first.
with anti-Flag antibody and second with the PPS-specific antibody APA1/1. The asterisk indicates the presence of a protein fragment in lane 1 that corresponds to the partial proteolysis of Flag-CD3ε, probably caused by cellular proteases contained in the cell lysate. The balance sheet indicates the percentage of protected CD3ε molecules, calculated by densitometry of the anti-Flag APA1/1 immunoblots. (C) The CD3ε tails become protected from proteolytic cleavage after TCR triggering. A Jurkat cell lysate was incubated with 10 μg/ml of either OKT3 or an isotypic control antibody (OKT8) before digestion with trypsin. Immunoblotting was performed with anti-CD3ε antibodies reacting with the C-terminus (448) or the whole tail (anti-ε, cit). (D) Tyrosine phosphorylation does not affect the sensitivity of CD3ε to trypsin proteolysis. A Jurkat cell lysate was incubated with 10 μg/ml of OKT3 in the presence of 20 μM of the src kinase inhibitor PP2 for 15 min at room temperature, before digestion with trypsin. Immunoblotting was performed with antibody APA1/1. (E) Protection against trypsin proteolysis is independent of TCR aggregation. A Jurkat cell lysate was preincubated with 50 μg/ml of Fab fragments of OKT3 or the isotypic control antibody 12Ca5 (anti-HA), before digestion for 15 min at 57°C with the indicated concentrations of trypsin. Immunoblotting was performed with APA1/1 followed by reprobing with the 448 antiserum. The presence of immunoglobulin light chain (L) from the anti-HA Fab fragment in the APA1/1 immunoblot is indicated.

Results

The CD3 tails adopt a compact conformation upon TCR engagement

We have previously reported that in a pull-down assay immobilized glutathione S-transferase (GST)-Nck binds to stimulated TCR complex but not to the non-stimulated complex [17]. Since Nck binds through its N-terminal SH3 domain to the PPS of CD3ε, the pull-down assay revealed a rearrangement of the CD3ε tail. The assay indicated that the non-stimulated TCR complex was in a non-binding conformation, which we called closed-CD3ε [33]. This form was converted upon stimulation into a binding conformation, which we called open-CD3ε. This conformational change was also suggested by positive immunostaining of stimulated cells and tissues with the CD3ε’s PPS-specific monoclonal antibody APA1/1 [34,35]. Thus, the pull-down and APA1/1 staining assays both demonstrated that the PPS of CD3ε becomes exposed after TCR engagement.

To further define ligand-induced conformational change in TCR cytoplasmic tails, and also to study whether CD3 subunits other than CD3ε are affected, we performed a series of protease-sensitivity assays. Since the tail of CD3ε is rich in lysine and arginine residues that are recognized as cleavage sites by trypsin (Fig. 1A), we chose this protease for our studies. To allow examination of the products of C-terminal-end digestion, experiments were done in Jurkat T cells stably expressing a human CD3ε chain labeled at its N-terminus with a Flag epitope (ε-Jk cells). Trypsin digestion of detergent lysates of ε-Jk cells generated a CD3ε partial-digestion product of 19 kDa, representing a loss of 4 kDa, which is most of the CD3ε tail (Fig. 1B). The partial digestion product was not recognized on immunoblots by the PPS-specific antibody APA1/1, suggesting that this sequence had been degraded. Digestion was, however, partially inhibited by addition to the lysate of the stimulatory anti-CD3 antibody OKT3. In contrast, the anti-Flag antibody did not inhibit digestion. Anti-Flag stimulation of ε-Jk cells was previously demonstrated, using the pull-down assay, to be a poor inducer of the conformational change in the TCR, even though it induced tyrosine phosphorylation (Fig. S1). The OKT3-protected CD3ε band was recognized by APA1/1, suggesting that the PPS was protected (Fig. 1B). These results indicate that binding of a conformational-change-inducing antibody to the TCR complex renders the CD3ε resistant to trypsin digestion, providing further support that the tail of CD3ε undergoes a conformational change in response to engagement by a stimulatory antibody.

To determine whether the CD3ε subunit also undergoes conformational change, trypsin-digested Jurkat T cell lysates were immunoblotted with antibody 448, which is specific for the C-terminal-most 34 amino acids of CD3ε. This showed that the CD3ε dimer was digested by trypsin upstream of the sequence recognized by the antibody (Fig. 1C). Incubation with the stimulatory antibody OKT3, but not with an irrelevant isotype-matched antibody, partially prevented the loss of the 448 epitope. Furthermore, reprobing the membrane with a polyclonal antibody raised against the whole tail of CD3ε detected intermediate-sized partial-digestion products only in cell lysates that had been incubated with OKT3 (Fig. 1C, WB anti-ε, cit).

Since TCR triggering with the anti-CD3 antibody OKT3 was performed after lysis of the cells in detergent, it was unlikely that the protective effect on the CD3ε and CD3ε tails was due to shielding caused by tyrosine phosphorylation of the immune receptor tyrosine-based activation motifs (ITAMs) or to recruitment of signalling proteins such as ZAP70. Nevertheless, in order
to exclude this possibility, we included the potent Src family kinase inhibitor PP2 [36] in the protease sensitivity assay. The result showed that, even in the presence of PP2, the triggered TCR acquired resistance to trypsin digestion (Fig. 1D), suggesting that the protection effect of TCR triggering was not due to a post-lysis modification of the TCR. Another important control was to demonstrate that the acquisition of resistance to trypsin was not due to the formation of aggregates that are poorly accessible to trypsin upon crosslinking of the TCR with the bivalent anti-CD3 antibody. Engagement of the TCR complex in Jurkat cell lysates with a monovalent Fab fragment of OKT3, but not the incubation with an isotype-matched irrelevant antibody, increased the resistance of the CD3e and CD3ζ tails to trypsin digestion (Fig. 1E). This result indicates that the acquisition of resistance to trypsin digestion is not caused by aggregation of the TCR, and is consequent with previous evidence showing, with the pull-down assay, that a monovalent anti-CD3 antibody induces the conformational change [17]. The results shown in Figures 1D and 1E exclude alternate explanations, and suggest that the acquisition of resistance to trypsin digestion is caused by a conformational change transmitted to the CD3 tails.

The acquisition of resistance to trypsin digestion by CD3e and CD3ζ was also noted when OKT3 was used to stimulate intact Jurkat T cells before lysis, but not when an irrelevant antibody (anti-CD4) was used (Fig. 2A). Furthermore, the protected CD3e and CD3ζ bands correspond to the full-length proteins, since they were recognized by M20 and 448, respectively, two polyclonal antibodies specific for the C-terminal ends. This result suggests that the conformational change involves a rearrangement of the whole tails.

The protective effect of TCR stimulation against trypsin digestion of CD3e and CD3ζ tails was seen not only with anti-CD3 dimer antibodies (e.g. OKT3), but also with stimulatory antibodies for the TCRζ/β heterodimer. Thus, stimulation of intact Jurkat cells with antibodies C305 and BV8, specific for the variable Vβ region, partly prevented the degradation of the CD3e and CD3ζ tails by trypsin (Fig. 2B). This effect was, however, not seen with the anti-Cβ antibody Jovi.1, which is a poor inducer of the conformational change according to the GST-Nck pull-down assay [17] and Fig. 2C). Isotypic differences cannot explain the differential effect of the anti-TCR antibodies, at least for antibodies OKT3, Jovi.1 and HP2/6 (irrelevant antibody in Fig. 2C, lane 2) which are of the same isotype (IgG2a). These results strongly suggest that TCR engagement induces a conformational change that is transmitted to the cytoplasmic tails of CD3e and ζ.

From the mobilities of the digestion products (Fig. 1A and 1C) it appeared that the cytoplasmic tail of CD3e from non-triggered TCRs was completely digested, whereas the tail of CD3ζ was only partly accessible to trypsin. The estimated loss of relative mass in the CD3ζ dimer after digestion was 16 kDa (from 32 to 16 kDa), which is below the 24 kDa loss that would be expected if all the CD3ζ tail were digested (Supplemental material Fig. S2). Upon stimulation, partial proteolytic products were detected at 22 and 27 kDa (Fig. 1C). The sequences that separate the three Immune receptor Tyrosine-based Activation Motifs (ITAM) in CD3ζ are particularly rich in basic amino acids, and therefore in potential trypsin-cleavage sites (Fig. 1A, 3A). Inspection of ITAM distribution in CD3ζ suggests that the 27 kDa fragment could derive from a cleavage between ITAMs B and C, and the 22 kDa fragment from cleavage within ITAM B (Suppl. Fig. S2). The 16 kDa product resulting from digestion of CD3ζ in resting TCRs could derive from cleavage between ITAMs A and B. If these calculations are correct, they would indicate that compared with the more membrane-distal ITAMs, ITAM A might be constitutively protected from trypsin attack in the resting TCR. No antibody specific for ITAM A was available, so to test this we generated a truncated CD3ζ mutant with a Flag epitope appended immediately after ITAM A (Fig. 3A, construct AFlag). Transfection of this construct into Jurkat cells generated disulfide-linked
Homodimers and heterodimers of Flag and endogenous CD3ζ underwent the conformational change after TCR triggering, as indicated by a positive reaction in the GST-Nck pull-down assay (Fig. 3B). However, Flag was completely resistant to trypsin digestion even in non-triggered TCRs, whereas the Flag epitope was completely digested when appended at the C-terminal end of full-length CD3ζ (Fig. 3C). These results suggest that the CD3ζ ITAM closest to the membrane is permanently protected from digestion and that the conformational change in the TCR modifies the exposure of the second and third ITAMs of CD3ζ to trypsin cleavage.

**Discussion**

Limited proteolysis has become an established tool for the study of conformational changes [37,38]. For instance, the atrial natriuretic peptide receptor becomes susceptible to cleavage by exogenously added protease when bound to its ligand [38]. In this paper we have used limited proteolysis to study the induction of conformational changes in the TCR complex. Our results show that the cytoplasmic tails of CD3ζ and CD3ζ subunits extracted from resting cells are almost completely digested by added trypsin, but become protected upon TCR triggering. Up to now, the strongest evidence for a conformational change in the TCR has come from studies showing ligand-induced exposure of the PPS in the cytoplasmic tail of CD3ζ [17]. One biochemical assay is based on pull-down with immobilized GST-Nck, and has served to demonstrate that certain stimulatory anti-CD3 antibodies [17] and a panel of pMHC ligands [33] induce a conformational change in the TCR. A second assay to detect these conformational changes is based on exposure of a neo-epitope recognized by the monoclonal antibody APA1/1. This epitope coincides with the PPS in CD3ζ and reveals, like the pull-down assay, a conformational change transmitted to the cytoplasmic tail of CD3ζ [34]. Through the use of APA1/1 it has been possible to confirm that the TCR undergoes conformational changes during antigen recognition in vivo, and that the TCR complexes undergoing the conformational change...
Figure 4. Model of conformational change in the cytoplasmic tails of CD3 subunits after TCR engagement. In non-stimulated cells, the CD3 subunits in the TCR are in a loose conformation that is accessible to trypsin digestion. The PPS of CD3ε (pink square) is in a non-binding conformation for Nck. After TCR engagement with pMHC or with stimulatory antibodies, the CD3 ectodomains adopt an active conformation (symbolized with rectangular forms) that is transmitted to the cytoplasmic tails of the CD3 subunits via a rotation and/or scissor movement. The cytoplasmic tails close up to form a compact structure that is less sensitive to proteolytic attack. This conformation permits Nck-binding by CD3ε.

The conformational change in the cytoplasmic tail of CD3ε was based on in vitro biochemical studies. Isolated CD3ε cytoplasmic tails change from a lipid-bound helical structure to an unfolded conformation upon tyrosine phosphorylation [29]. These results support the earlier finding that a synthetic peptide corresponding to the third ITAM of CD3ε adopts an α-helical structure in the non-phosphorylated form and a β strand conformation when phosphorylated [39]. However, both these studies were performed in cell-free systems with synthetic peptides and recombinant proteins, in the absence of other TCR subunits. The limited proteolysis studies reported in this paper therefore provide the first evidence that CD3ε undergoes a conformational change within the TCR complex in response to ligand binding. Due to the lack of appropriate antibodies, we have not yet been able to study the effect of limited proteolysis on CD3γ and CD3δ, but a model could be proposed in which a conformational change is transmitted from the ligand-binding ectodomains of the TCRα/β to the cytoplasmic tails of all CD3 subunits.

The conformational change induced in CD3ε, however, shows distinct features from those confirmed for CD3ζ. Whereas the whole cytoplasmic tail of CD3ζ is susceptible to proteolytic degradation in the non-triggered TCR and is protected upon stimulation, in CD3ε ITAM A is protected even in non-triggered TCRs, and stimulation extends this protection to ITAMs B and C. Interestingly, CD3ζ is sequentially phosphorylated on its three ITAMs during T cell activation [for a review see [40]]. In some cases ITAMs B and C are phosphorylated constitutively, producing the p21 tyrosine phosphorylated form of CD3ζ. In contrast ITAM A is phosphorylated only upon TCR triggering, yielding the p23 form. Although the detailed functional significance of the p21 and p23 forms of CD3ζ is not clear, it is well-established that phosphorylation of ITAM A defines the difference between triggered and non-triggered TCRs. In light of our limited proteolysis results, we suggest that, in a resting TCR, ITAM A is in a compact conformation that is not accessible to tyrosine phosphorylation by the priming src kinases [41], and that the conformational change not only compacts ITAMs B and C, but also reorients ITAM A into a conformation more susceptible to phosphorylation.

We recently described that both TCR crosslinking and conformational change are required for full tyrosine phosphorylation of different intracellular effectors [30]. In this regard, the acquisition of protease resistance by the tail of CD3ε shown in the present study suggests that the conformational change may also affect the phosphorylation of CD3ζ and, therefore, the recruitment of ZAP70 and the subsequent phosphorylation of downstream effectors. This requirement of the conformational change for tyrosine phosphorylation appears to be contradicted by the activation of tyrosine phosphorylation with the anti-flag antibody, a poor inducer of the conformational change in Flag-CD3ε-expressing Jurkat cells (Supplemental Figure S1). Several explanations can be given. The most simple is that the anti-flag antibody induces the conformational change to a level that is sufficient to pass a threshold for activation of tyrosine kinases. A second possibility, is that TCR crosslinking in the absence of conformational change is sufficient for the activation of tyrosine kinases but not for a normal pattern of phosphorylation, i.e. TCR-associated tyrosine kinases could be activated without the TCR undergoing a conformational change, but the access to their potential substrates (ITAMs and downstream effectors) would be limited. Finally, the number of tools that we have to study the conformational change, or conformational changes, in the TCR is limited. The exposure of the PPS in CD3ε, revealed by the GST-Nck pull-down assay and APA1/1 epitope display [17,34], and the trypsin sensitivity assay shown in the present study, might be only coarse methods to understand the fine tuning of signal transduction by the TCR.

The acquisition of resistance to trypsin digestion upon TCR stimulation suggests that, contrary to our prior prediction, stimulation does not shift the cytoplasmic tails of the TCR from a “closed” to an “open” conformation [17,33]. Our present data indicate instead that the cytoplasmic tails in the non-engaged TCR are in a loose conformation that makes them accessible to trypsin digestion. We now prefer to name this conformation the Nck non-binding or loose conformation. Upon antibody stimulation, a conformational change is transmitted from the TCR ectodomains to the CD3 cytoplasmic tails, which become packed into a more compact structure with reduced accessibility for trypsin. This is the Nck-binding or locked conformation. The reduced exposure of the CD3 cytoplasmic tails to trypsin must occur simultaneously with an increased exposure of the CD3ε PPS to Nck perhaps by fixing it into a conformation adequate for binding. We do not know the ultimate causes that explain why a trypsin-sensitive loose conformation in CD3ε is incompatible with a Nck-binding conformation of the PPS. We have however structural information [obtained by NMR on how the PPS binds the SH3.1 domain of Nck (Borroto and Alarcón, in preparation)]. The CD3ε polypeptide makes an extensive fingerprint on the SH3.1 domain, where not only the central proline residues participate, but also upstream and downstream charged amino acids. This explains why CD3ε binds with abnormally high affinity for a SH3-ligand interaction (in the order of 0.1 μM), and may also indicate structural requirements for the interaction. In the loose conformation the sequences upstream and/or downstream of the PPS-and not necessarily the central prolines themselves-might be in non-binding conformation. The compaction of the CD3 tails resulting in the locked conformation is further stabilized through mutual exclusion of the ends of the cytoplasmic tails.
conformation may bring the Nck-binding sequence into the appropriate conformation.

In this study we have used anti-CD3 antibodies to elicit the conformational change that results in the trypsin-protected or locked conformation in both CD3ε and CD3ζ. The locked conformation was also induced when antibodies that recognize the variable domain of the TCRβ ectodomain of the TCRζ/β heterodimer to the CD3 tails induces their locked conformation. We have attempted to demonstrate that a similar rearrangement takes place upon antigen stimulation. However these experiments have failed, probably due to the fact that continued pMHC-TCR interaction after lysis of the cells is necessary to preserve the conformational change [30].

The transmission of TCR conformational change across the plasma membrane presents a conceptual challenge. The structure of the CD3γ-CD3ζ dimer ectodomains led to the proposal that the transmembrane domains of the dimer undergo a piston-like movement. In this way, the paired G beta strands of CD3ε and CD3ζ (as well as those of CD3ε and CD3δ) would form a rigid rod-like connector that would displace the transmembrane helices [42]. On the other hand, monovalent Fab fragments of the anti-CD3 antibody OKT3 bind to CD3ε in a side-on orientation [43], and this interaction induces a conformational change in the TCR [17]. Considering this in conjunction with the electrostatic properties of CD3ε, it has been proposed that the transmission of the conformational change might require a rotational or scissor-like movement of both the transmembrane domains and the cytoplasmic tails of the CD3 subunits [43]. The protease-sensitivity data reported in the present paper support a model in which ligand binding to the TCRζ/β heterodimers somehow promotes a combination of rotational and closing-scissor movements that simultaneously condense and shield the cytoplasmic tails of the CD3 subunits, while exposing key features, such as the PPS of CD3ε and ITAM A of CD3ζ. Studies with purified recombinant proteins have shown that all ITAM-bearing cytoplasmic tails studied (including those of the TCR and BCR) form oligomers in solution [44], although the interaction is weak (Kd in the order of 10 μM). The conformational change in the TCR might exploit this natural propensity of ITAM-bearing cytoplasmic tails to dimerize or oligomerize, by forcing the local effective ITAM concentration above the dissociation constant. Although the capacity of the three ITAMs of CD3ζ to homodimerize has not been measured on a one-by-one basis, it is tempting to speculate that the protease resistance of ITAM A could be caused by a higher propensity of this ITAM to dimerize in the resting TCR, compared to ITAMs B and C. Alternatively, protease resistance of ITAM A in the resting TCR could be due to the position of CD3ζ in the TCR complex, i.e. the tail of CD3ζ could occupy an internal position within the TCR complex, where the tails of the other CD3 subunits could shield the membrane most proximal region of CD3ζ’s tail.

In our current model, the conformational change is initiated after binding of a cluster of two or more pMHC agonists to two or more TCRζ/β heterodimers within a multivalent TCR complex [30]. This would generate a torque on the TCRζ/β heterodimers that could be transmitted to the CD3 dimers. In turn, this binding would induce the rotation or sliding of the transmembrane domains of the CD3 dimer with respect to those of the TCRζ/β heterodimers, and an ensuing transmission of this movement to the CD3 cytoplasmic tails. Crystal structures of TCRζ/β ectodomains bound to pMHC complexes almost universally show that the orientation of TCRζ/β is approximately diagonal to the MHC peptide-binding groove (for a review see [45]). We hypothesize that in the context of a multivalent TCR, the diagonal orientation imposed by pMHC binding on the two TCRζ/β heterodimers may be responsible for the torque transmitted to the CD3 subunits.

### Materials and Methods

**Plasmids**

The pGEX-4T1 derivative GST-SH3.1, containing the amino-terminal SH3 domain of Nck, was kindly provided by Dr. R. Geha (Children’s Hospital, Harvard Medical School, Boston). The pSRz-CD3ζ-Flag plasmid encoding human CD3ζ was generated by PCR. The truncated CD3ζA construct (pSRz-CD3ζA-Flag) expresses a protein truncated immediately after the first ITAM of human CD3ζ. Both constructs encode the Flag peptide fused to the C-terminus of the protein.

**Cells**

The human T cell line Jurkat was maintained in complete RPMI 1640 supplemented with 10% fetal bovine serum (Sigma). The Jurkat cell clone fi-jk expressing Flag-CD3ε has been described previously [17]. The JkεAF and JkεF cell lines were generated by stable transfection into Jurkat of the pSRz-CD3ζA-Flag and pSRz-CD3ζ-Flag, respectively.

**Antibodies**

The anti-CD3ε mouse monoclonal antibody APA1/1 has been described previously [34,35,46,47], as has the rabbit anti-CD3ζ antisera 448 [48,49]. The anti-CD3ζ/cit rabbit antisera was obtained by repeatedly immunizing a white New Zealand rabbit with a purified peptide corresponding to the complete cytoplasmic tail of human CD3ζ, produced in E.coli. The anti-CD3ε/mAb Jovi.1 mAb (IgG2a) and the anti-CD8 mAb 24/3 (IgG2a) were a kind gift of Dr. M. Owen (CRUK, London, UK) and Dr. F. Sánchez-Madrid (Hospital de La Princesa, Madrid). Hybridomas producing the anti-CD3 mAb OKT3 (IgG2a), the anti-CD3 mAb OKT8 (IgG2a) were obtained from ATCC. Goat anti-CD3ε m20, mouse anti-human Vβ BV8 (IgG2b), mouse anti-hemagglutinin (HA) 12CA5 (IgG2a), and mouse anti-Flag (M2) antibodies were purchased from Santa Cruz Biotechnology, BD-Pharmingen, Roche and Sigma, respectively. Fab fragments were prepared using the Immunoopure IgG1 Fab Preparation kit (Pierce) and confirmed by SDS-PAGE and silver staining.

**Stimulation and pull-down assay**

10⁵ cells per point were collected, washed, resuspended in RPMI and incubated without serum at 37°C for 2 h. Cells were stimulated with 10 μg/ml soluble antibody for 5 min at 37°C and lysed in 20 mM Tris-HCl pH 8.2, 0.3% Brij96, 140 mM NaCl, 10 mM iodoacetamide and 2 mM EDTA (lysis buffer), plus 1 μg/ml each of leupeptin and aprotinin, and 1 mM phenyl methyl sulfonyl fluoride (PMSF). For GST pull-down assays supernatants were first precleared with GST adsorbed to glutathione-Sepharose (Amersham Biosciences) before precipitation with GST-SH3.1 protein adsorbed to glutathione-Sepharose [17].

**Trypsin digestion**

10⁵ cells per point were stimulated with 10 μg/ml soluble antibody for 5 min at 37°C and disrupted in lysis buffer without leupeptin, aprotinin or PMSF. A total of 1 μg/ml trypsin (Sigma) was added to the lysate and the reaction mix was incubated for 15 min at 37°C. Afterwards, 3× Laemmli’s sample buffer was added.
added, and samples boiled, to stop the enzyme activity. Alternatively, postnuclear cell lysates of unstimulated cells were incubated with the anti-CD3 antibody before trypsin digestion under the same conditions.

Supporting Information

Supplemental Figure S1 TCR crosslinking with an antibody to a foreign CD3 epitope results in poor stimulation. (A) Anti-Flag stimulation of f epsilon-Jk cells is a weak inducer of the conformational change, but strong inducer of tyrosine phosphorylation. Jurkat T cells transfected with Flag-CD3epsi (fepsilon-Jk) cells were stimulated with the indicated concentrations of an anti-Flag or anti-CD3 antibody (OKT3), lysed in Brij96, and TCR binding to GST-SH3.1 was revealed by immunoblotting with anti-CD3zeta antibody. (B) Compared to anti-CD3, the anti-Flag antibody poorly activates T cells. The expression of CD69 and CD25 was examined in f epsilon-Jk cells 24 h after stimulation with OKT3 (closed symbol) or anti-Flag (open symbol). Induction of programmed cell death was examined 48 h after stimulation with immobilized antibodies by propidium iodide exclusion (PI staining) or by cell cycle analysis (% cells in sub-G1).

Found at: doi:10.1371/journal.pone.0001747.s001 (0.84 MB TIF)

Supplemental Figure S2 Expected and observed sizes of partial trypsin digestion of the CD3zeta tail. A cartoon of the CD3epsi and CD3zeta subunits showing potential tryptic cleavage sites (arrows). Transmembrane domains are indicated with grey boxes, and the relative positions of the three ITAMs are marked. The observed sizes of the partial digestion products were calculated from results shown in Fig. 1C.

Found at: doi:10.1371/journal.pone.0001747.s002 (3.46 MB TIF)

Acknowledgments

We thank Drs. Simon Bartlett and Hise M. van Santen for critical reading of the manuscript.

Author Contributions

Conceived and designed the experiments: BA. Performed the experiments: RR. Analyzed the data: WS. Wrote the paper: BA.

References

1. Hubbard SR, Till JH (2000) Protein tyrosine kinase structure and function. Annu Rev Biochem 69: 373–390.
2. Weiss A, Schlessinger J (1998) Switching signals on or off by receptor dimerization. Cell 94: 277–280.
3. Wess J (1997) G-protein-coupled receptors: molecular mechanisms involved in receptor activation and selectivity of G-protein recognition. FASEB J 11: 346–354.
4. Wess J, Liu J, Blin N, Yan J, Leche C, et al. (1997) Structural basis of receptors G protein coupling selectivity studied with muscarinic receptors as model systems. Life Sci 60: 1007–1014.
5. Jiang G, Hunter T (1999) Receptor signaling: when dimerization is not enough. Curr Biol 9: R561–571.
6. Chan FK, Chun HJ, Zheng L, Siegel RM, Bui KL, et al. (2000) A domain in TNF receptors that mediates ligand-independent receptor assembly and signaling. Science 280: 2351–2354.
7. Chan FK, Siegel RM, Zacharias D, Swolford R, Holmes KL, et al. (2001) Fluorescence resonance energy transfer analysis of cell surface receptor interactions and signaling using spectral variants of the green fluorescent protein. G proteom 44: 361–368.
8. Remy I, Wilson EA, Michnick SW (1999) Erythropoietin receptor activation by a ligand-induced conformation change. Science 283: 990–993.
9. Siegel RM, Frederiksen JK, Zacharias DA, Chan FK, Johnson M, et al. (2000) Fas preassociation required for apoptosis signaling and dominant inhibition by pathogenic mutations. Science 288: 2354–2357.
10. Tolar P, Sohn HW, Pierce SK (2005) The initiation of antigen-induced B cell antigen receptor signaling viewed in living cells by fluorescence resonance energy transfer. Nat Immunol 6: 1160–1167. Epub 2005 Oct 1162.
11. Liu J, Weiss A (2000) T cell receptor signalling. J Cell Sci 114: 241–244.
12. Rudolph MG, Luz JG, Wilson IA (2002) Structural and thermodynamic correlates of T cell signaling. Annu Rev Biophys Biomol Struct 31: 121–149.
13. Werlen G, Palmer E (2002) The T-cell receptor signalosome: a dynamic structure with expanding complexity. Curr Opin Immunol 14: 299–305.
14. Alarcon B, Gil D, Delgado P, Schamel WW (2003) Initiation of TCR signaling: a conformation- and avidity-based proofreading mechanism for the TCR-CD3 complex. Trends Immunol 24: 189–195. Epub 2003 Feb 2024.
15. Gil D, Schrum AG, Alarcon B, Palmer E (2003) T cell receptor engagement by peptide-MHC ligands induces a conformational change in the CD3 complex of thymocytes. J Exp Med 201: 517–522.
16. Ruisenor RM, Gil D, Fernandez E, Sanchez-Madrid F, Alarcon B (2005) Ligand-induced conformational changes in the T-cell receptor associated with productive immune synapses. Blood 106: 601–608. Epub 2005 Mar 2024.
17. Ruisenor RM, van Santen HM, Alarcon B (2006) A conformational change is not enough for T cell receptor-ligand interaction during thymic selection. Proc Natl Acad Sci U S A 103: 9625–9630. Epub 2006 Jun 9629.
18. Hanke JH, Azad MT, Winkel F, Hamp J, Reich Z, et al. (1998) Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. J Biol Chem 273: 695–701.
19. Gilbert JM, Hernandez LD, Ballew J, Bates P, White JM (1995) Receptor-induced conformational changes in the subgroup A avian leukosis and sarcoma virus envelope glycoprotein. J Virol 69: 7410–7415.
20. Hsu X, Ata T, Minouo KS (1999) Ligand-binding-dependent limited proteolysis of the aortic natriuretic peptide receptor: juxtaparameter hinge structure essential for transmembrane signal transduction. Biochemistry 38: 16951–16955.
21. Leczko I, Holliss M, Vass E, Hegedu G, Monosto E, et al. (1998) Conformational effect of phosphorylation on T cell receptor/CD3 zeta-chain sequences. Biochem Biophys Res Commun 242: 474–479.
40. Pitcher LA, Young JA, Mathis MA, Wragg PC, Bartok B, et al. (2003) The formation and functions of the 21- and 23-kDa tyrosine-phosphorylated TCR zeta subunits. Immunol Rev 191: 47–61.

41. Malissen B (2003) An evolutionary and structural perspective on T cell antigen receptor function. Immunol Rev 191: 7–27.

42. Sun ZJ, Kim KS, Wagner G, Reinherz EL (2001) Mechanisms contributing to T cell receptor signaling and assembly revealed by the solution structure of an ectodomain fragment of the CD3 epsilon gamma heterodimer. Cell 105: 913–923.

43. Kjer-Nielsen L, Dunstone MA, Kostenko L, Ely LK, Beddoe T, et al. (2004) Crystal structure of the human T cell receptor CD3 epsilon gamma heterodimer complexed to the therapeutic mAb OKT3. J Biol Chem 279: 12807–12816.

44. Sigalov A, Aivazian D, Stern L (2004) Homooligomerization of the cytoplasmic domain of the T cell receptor zeta chain and of other proteins containing the immunoreceptor tyrosine-based activation motif. Biochemistry 43: 2049–2061.

45. Rudolph MG, Stanfield RL, Wilson IA (2006) How TCRs bind MHCs, peptides, and coreceptors. Annu Rev Immunol 24: 419–466.

46. Alarcon B, Ley SC, Sanchez-Madrid F, Blumberg RS, Ju ST, et al. (1991) The CD3-gamma and CD3-delta subunits of the T cell antigen receptor can be expressed within distinct functional TCR/CD3 complexes. Embo J 10: 903–912.

47. Borroto A, Mallababarra A, Albar JP, Martinez AC, Alarcon B (1998) Characterization of the region involved in CD3 pairwise interactions within the T cell receptor complex. J Biol Chem 273: 12807–12816.

48. Sahuquillo AG, Roumier A, Teixeiro E, Bragado R, Alarcon B (1998) T cell receptor (TCR) engagement in apoptosis-defective, but interleukin 2 (IL-2)-producing, T cells results in impaired ZAP70/CD3-zeta association. J Exp Med 187: 1179–1192.

49. San Jose E, Sahuquillo AG, Bragado R, Alarcon B (1998) Assembly of the TCR/CD3 complex: CD3 epsilon/delta and CD3 epsilon/gamma dimers associate indistinctly with both TCR alpha and TCR beta chains. Evidence for a double TCR heterodimer model. Eur J Immunol 28: 12–21.