Brief Definitive Report

Signaling Efficiency of the T Cell Receptor Controlled by a Single Amino Acid in the β Chain Constant Region

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Summary

A single amino acid residue, Gln₁₃₆, located within the connecting peptide domain of Cβ controls the ability of the α/β TCR to transmit a full signal. TCRs in which this Cβ residue is mutated to Phe, the residue found in TCR-γ, are unresponsive to antigenic ligands. Interestingly, this Cβ residue is either polar or charged in every species studied thus far, including the trout and the skate. In contrast, the analogous residue in Cγ is always hydrophobic. In spite of their compromised antigen responsiveness, the mutant TCR complex contains the CD3-γ, -δ, -ε, and -ζ chains, and undergoes ζ chain phosphorylation and ZAP-70 recruitment. However, the biological response of the mutant TCR could be rescued with a calcium ionophore, implying that mutant TCRs are defective in generating a calcium-mediated signal. The implications of the differences between Cβ and Cγ are considered.

One of the primary goals of immunologists over the last 10 yr has been to understand how the TCR transduces a signal (1). The TCR complex consists of an antigen-binding α/β heterodimer supported by noncovalently associated CD3-γ, -δ, and -ε as well as ζ chains (2). The α and β constant regions are presumably required to propagate a signal to the CD3 and ζ components of the complex. Considered together, the cytoplasmic tails of the CD3 and ζ chains contain 10 immunoreceptor tyrosine-based activation motifs (ITAMs), which are rapidly phosphorylated upon TCR engagement (3, 4). Subsequently, the protein tyrosine kinase ZAP-70 is recruited to the TCR complex and activated (5, 6). Thereafter, large multimolecular complexes of proteins participating in the signaling cascades assemble around the cytoplasmic domains of activated TCRs (7), which leads to the initiation of intracellular signaling pathways.

Although homologous to its α/β relative, the γ/δ TCR is expressed in a separate lineage of T lymphocytes (8). While it has been generally assumed that both classes of TCR use similar mechanisms to generate a signal, this may not be the case, as there are several features that distinguish these two types of T cell receptors. The CD3-ε chains contain within human α/β receptors an epitope which is masked in γ/δ receptors (9, 10). Furthermore, unlike α/β receptors, γ/δ TCRs can be expressed in the absence of CD3-δ, can develop in MHC deficient mice, and can recognize unprocessed antigens in the absence of MHC presentation (11–14). Finally, the TCR-γ chain contains a motif within its connecting peptide (CP) domain that is required for the transduction of antigenic signals (15). α/β TCRs lacking this α chain-connecting peptide motif (α-CPM) do not properly interact with the CD3 complex and fail to generate Ca²⁺-mediated signals. α-CPM is specific for the α/β TCR and seems to have evolved after the divergence of TCR-α and -δ chains. This motif is found within α/β but not γ/δ TCRs implies that these two types of TCRs may use subtly different signaling mechanisms.

We wondered whether there exists a region within the TCR-β chain, analogous to the α-CPM, which is required to produce a functional α/β receptor. The experiments reported here identify a single amino acid within the β chain-CP domain that controls TCR signaling efficiency. Mutation of this β chain amino acid to a residue commonly seen in TCR-γ chains generates an α/β receptor which is particularly inefficient in transducing signals from antigenic ligands.

Materials and Methods

Generation of DNA Constructs. The Vα2.1 and Vβ8.1 TCR cDNAs were isolated from the T cell hybridoma, 3BBM 74, and confer reactivity to the I-A^{b/noll} alloantigen and the staphylococcal enterotoxin B (SEB) superantigen (16). The wild-type (wt) and mutated TCR constructs were generated using overlapping oligo nucleotides and PCR, as previously described (15). All constructs were verified by DNA sequencing using the SequiT therm™ cycle sequencing kit (Epitrend Technologies Corp., Madison, WI) and the deduced amino acid sequences of the mutant β chains are shown in Fig. 1.

Production of Retroviral Supernatants and Infection of T Cell Hybridoma. Retroviruses encoding wt or chimeric TCR chains were generated and used to infect the 58hCD4, as previously described (15).
Cell Lines. The 58B.CD4 cell line has been previously described (15, 17) and was provided by O. Acuto (Pasteur Institute, Paris, France). The murine fibroblast cell line DAP.3 transfected with human HLA class II DR1 molecules (18) or the human, DR1-expressing, B lymphoblastoid cell line 221 (19) was used to present SEB. The use of the cytokine indicator cell lines HT-2 and FDC-P1 have been previously described (15).

Antibodies. The use of anti-VO, anti-CD3, anti-Vβ8.1 (F23.1), and anti-CD3-ε (145-2c11) mAbs, as well as anti-CD3-γ, anti-CD3-δ, and anti-CD3-ε rabbit antisera have been previously described (20). The anti-δ chain mAb, H146-968 (21), was purified from culture supernatants using protein G-Sepharose beads (Pharmacia Biotech AB, Uppsala, Sweden). The rabbit anti-ZAP-70 antiserum was provided by Dr. L. Samelson (National Institutes of Health, Bethesda, MD). The antiphosphotyrosine mAb 4G10 was purchased from Upstate Biotechnology Inc. (Lake Placid, NY).

Flow Cytometry, T Cell Stimulation, and IL-2 and IL-3 Assays. The levels of cell-surface antigens were detected by immunofluorescence and analyzed on a FACScan using the CELLQuest analysis software (Becton Dickinson, Rutherford, NJ). T cell stimulation and IL-2 and IL-3 assays were performed as previously described (15).

Immunoprecipitation and Western Blotting. T hybridoma cells (2 × 10⁷/sample) were lysed in buffers containing 1% digitonin and the TCR complex immunoprecipitated with 2 μg of B20.1 (anti-VO). Immunoprecipitations, Western blotting, and detection of CD3 chains were carried out as previously described (20).

ζ Chain Tyrosine Phosphorylation Analyses. For superantigen stimulation, 10⁶ APCs were incubated with or without SEB (10 μg/ml) for 2 h in 1 well of a 24-well plate. 10⁷ T hybridoma cells were added to the APCs, centrifuged at 1,000 rpm for 30 s, and then incubated at 37°C for 20 min. Cells were washed twice with ice cold PBS containing 0.4 mM Na₃VO₄ and 0.4 mM EDTA, and were then lysed in 0.5 ml lysys buffer (10 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotenin) containing 2 mM Na₃VO₄, 25 mM NaF, and 1% Triton X-100. Lysates were centrifuged at 12,000 g for 10 min, and the relevant proteins in the supernatant were immunoprecipitated with 1.5 μg of H146-968 (anti-ζ) mAb or 3 μl of a rabbit polyclonal anti-ZAP-70 antiserum. Immunoprecipitates were recovered using protein G-Sepharose beads. The tyrosine phosphorylated proteins present in these immunoprecipitates were analyzed by Western blotting and detected using the antiphosphotyrosine mAb 4G10.

Results

To better understand the functional relationships between the α/β heterodimer and the CD3 and ζ components, we made a number of chimeric TCR-β chains by replacing domains from the β chain constant region with homologous domains from the TCR-γ chain. We first generated a chimeric β chain, βVII, which contained TCR-β-derived V, D, J, and C region sequences up to and including the interchain Cys, followed by Cγ sequences encoding the COOH-terminal part of the CP domain, the transmembrane domain, and the cytoplasmic tail (Fig. 1 A). As seen in Fig. 1 B, this chimeric βVII chain was not expressed at the cell surface when paired with a wt α chain. However, Cβ and Cγ differ in the length of their CP domains. There are 13 amino acids between the interchains Cys²⁷ and Ala³⁴ in all β chains and 16 amino acids between Cys²⁷ and Ala³⁴ in all mammalian γ chains (Fig. 1 A and reference 22). Thus, the fact that the βVII chain could not be expressed at the cell surface might have been due to the possibility that the Cγ length in this region (16 amino acids) was incompatible with surface expression in the context of an α/β TCR.

To test this idea, we constructed two additional chimeras, βV and βVII, which respected the conserved β chain length (13 amino acids) in this region (αwt; Fig. 1 A). Both the βV and the βVII chimeras were expressed at the cell surface in conjunction with a wt α chain (αwt; Fig. 1 B). In fact, the ααwt/βV and the ααwt/βVII chimeric TCRs were not expressed at all expressed at two- to threefold higher levels on the cell surface than were the ααwt/βwt receptor. Hybridomas expressing these chimeric TCRs were stimulated with the superantigen SEB bound to APCs. As seen in Fig. 2 A, the ααwt/βV TCR responded to SEB about as well as the wt TCR, while the ααwt/βVII TCR was clearly less sensitive (~100-fold) to this superantigen. This signaling defect was seen even more clearly when the SEB response of a hybridoma expressing the ααwt/βVIII mutant receptor was compared to that of an ααwt/βVIII hybridoma sorted for an equivalently high level of TCR expression (Fig. 2 B). Furthermore, transgenic mice expressing the ααwt/βVII TCR were defective in responding to SEB and the I-Aβ113 alloantigen (data not shown). Thus, the signaling defect of this mutant TCR was not limited to superantigens. On the other hand, these chimeric TCRs were activated by plate-bound anti-TCR mAbs (Fig. 2 C and data not shown), indicating that the hybridomas expressing the ααwt/βVIII TCRs were not intrinsically defective. Therefore, TCRs comprised of the chimeric βVII chain seemed to be specifically deficient in transducing signals from antigenic ligands.

TCRs from these hybridomas were immunoprecipitated, and the associated polypeptides were analyzed by Western blotting (data not shown). The CD3-γ, -δ, and -ζ chains as well as the ζ polypeptides were coexpressed with hybridomas expressing either the wt TCR or the signaling-defective, ααwt/βVIII TCR (data not shown). In spite of a pronounced signaling defect (Fig. 2), the interactions with the CD3 and ζ chains were preserved in complexes containing the functionally defective, ααwt/βVIII heterodimer.

The tyrosine phosphorylation of the ζ chain was examined in these hybridomas as well. From the data in Fig. 3 A, it was apparent that the ζ chains in the ααwt/βwt and ααwt/βVIII TCRs could be tyrosine phosphorylated and that both forms (p21 and p23) of the phosphorylated ζ chain could be generated. Although the tyrosine phosphorylation of ZAP-70 could not be demonstrated even in hybridomas expressing the ααwt/βwt receptor (data not shown), an anti-ZAP-70 antiserum was used to evaluate ZAP-70 recruitment (Fig. 3 B). In superantigen-stimulated cells, expression of ZAP-70 could not be demonstrated even in hybridomas expressing the ααwt/βVIII TCR. In contrast, there was clear recruitment (Fig. 3 C). This difference was in incompatible with surface expression in the context of an α/β TCR.
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that the mutant TCR was defective or inefficient in activation in the presence of PMA or ionomycin. The impaired response of the wt TCR was likely down-stream from ZAP-70 recruitment.

Binding of an appropriate ligand to the TCR eventually leads to the activation of the protein kinase C/ras and the calcium intracellular signaling pathways (1, 7). Deficient activation of these pathways can be compensated for by the addition of a calcium ionophore, such as ionomycin used at several different concentrations of PMA, whereas defective activation of the calcium pathway can be compensated for by the addition of the phorbol ester, PMA, whereas deficient activation of the PKC/ras pathway can be compensated for by the addition of the phorbol ester, PMA.

The functional chimera, βVI, contains the β chain sequence ASYQQ, which has been replaced with the γ chain sequence, LQFQF, in the nonfunctional chimeric chain, βVI (Fig. 1 A). The most striking amino acids in this region are Tyr134 and Gln136, both of which are encoded by the wt chain cDNA, and the resulting mutant β chains were paired with a wt chain and tested for their activity to SEB. As seen in Fig. 5, A and B, the Tyr134 → Phe mutation had a negligible effect on the response to SEB, whereas TCRs carrying the Gln136 → Phe mutation had a marked and reproducible effect on SEB responsiveness. Considering the pronounced effect of the Gln136 → Phe exchange on superantigen responsiveness, we examined the amino acid residues present at this position in all CB and CG γ chains, some of which are shown in Fig. 5 C. Strikingly, the amino acids at this position in the β chain are always polar or charged. In contrast, γ chains always contain hydrophobic amino acids at this position (Fig. 5 C and reference 23).

Discussion

We took advantage of the differences between α/β and γ/δ TCRs to generate signaling-defective receptor mutants. Since the TCR-β and γ constant regions are structurally homologous, we could replace domains of CB with similar domains of CG. Most of the resulting chimeric chains paired with a wt α chain and were expressed on the cell surface (Fig. 1 B). Hybridomas expressing αwt/βVI TCRs are 100-fold less sensitive to antigenic signals than hybridomas expressing wt receptors (Fig. 2, A and B). The signaling defect seems specific for antigenic ligands, since αwt/βVI TCRs respond fully to mAbs capable of receptor cross-linking (Fig. 2 C and data not shown). What distinguishes the functional βV chimera from the defective βVI chimera are the amino acids present from position 132 to 136 that are derived from CB and CG, respectively (Fig. 1 A). Site-directed mutagenesis defined Glu136 as a critical residue in this region. This position has been conserved in the β chains of all known species from the skate to humans (23) and is either polar or charged (Fig. 5 C). In contrast, the analogous residue in the γ chain has been conserved as a hydrophobic amino acid (Fig. 5 C). This conservation is

Figure 1. Amino acid sequences and surface expression of chimeric TCR-βγ chains. (A) The sequences of the wt TCR-β chain (CB), wt TCR-γ chain (CG), and the 3 chimeric TCR-βγ chains (BV-B and CG) are shown using the single letter amino acid code. The boxes indicate the approximate boundary of the TM domain, defined using the Lasergene Navigator Protein Software program (DNASTAR, Inc., Madison, WI). (B) 58HCD4 (α/β) T cell hybridomas expressing wt α chains and wt β or wt/γB VI TCRs were stimulated with SEB in the presence of B23.1, and SAPE, and then analyzed by flow cytometry. Dashed lines represent fluorescence of the same cells stained with streptavidin-phycocerythrin alone. The solid vertical lines indicate the mean fluorescence intensity of wt/βVI TCR, ZAP-70 was nevertheless recruited to the phosphorylated γ chains. Thus, the signaling defect in hybridomas expressing this mutant TCR was likely downstream from ZAP-70 recruitment.

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functionally significant since changing this residue from a hydrophilic to a hydrophobic amino acid is accompanied by a significant loss in signaling efficiency (Figs. 2 and 5).

There is evidence that the \(\alpha/\beta\) and \(\gamma/\delta\) heterodimers do not interact with the CD3 complex in the same way. For example, \(\gamma/\delta\) TCRs can be expressed in the absence of CD3-\(\delta\) (11). Furthermore, a CD3-\(\epsilon\) epitope, contained within human \(\alpha/\beta\) receptors and recognized by the mAb WT31 is masked in \(\gamma/\delta\) receptors (9, 10, 24). Many of these differences might be attributed to the CP (membrane proximal) domains of the different TCR chains. We have previously identified a motif within the \(\alpha\) CP domain which is strikingly absent from TCR-\(\delta\) chains. This motif controls the proper association of CD3-\(\delta\) and -\(\zeta\) to the TCR complex and is required for antigen-driven signal transduction (15).

Figure 2. Response of chimeric TCRs to SEB and anti-TCR mAbs. In each experiment, \(5 \times 10^4\) 58hCD4 cells well expressing wt or chimeric TCRs were stimulated with \(2 \times 10^5\) DAP-3-DR1 cells and increasing doses of SEB (A and B) or plate-bound anti-V\(\beta8\) (C), and the culture supernatants were assayed for IL-2. Similar results were obtained using plate-bound anti-CD3-\(\epsilon\) and anti-V\(\alpha2\) mAbs (data not shown). Results shown are representative of two or more experiments.
Examining the CP domain of the β chain, we have found a region within Cβ which controls the efficacy of antigen-driven signal transduction. Although alteration of this region does not lead to changes in the subunit composition of the TCR complex (data not shown), a clear biological defect was observed (Fig. 2). Mutation of this region had no observable effect on the amount of ζ chain phosphorylation nor on the recruitment of ZAP-70 to the ζ homodimer (Fig. 3). Thus, the signaling defect in this mutant is likely downstream from ZAP-70 recruitment. The signaling defect can be corrected or at least compensated in the presence of a calcium ionophore (Fig. 4), suggesting that the mutation within the β chain may lead to the inefficient induction of Ca2+ mobilization.

There is little structural information available for the CP domains since this region has been truncated from the TCR heterodimers used in crystallographic analyses (25, 26). Nevertheless, the CP domains of the α and β chains may function as a unit since amino acids within these domains have been conserved in α/β TCRs during the last 500 million yr (15, 22, 23). The length of the CP domains has been conserved as well. Considering the region between the interchain Cys and the transmembrane domain (defined by the beginning of the CART motif [22]), all known β chains contain 13 amino acids (22, 23). On the other hand, α chains contain from 17 to 20 amino acids in this region. Thus, in α/β TCRs, a length disparity between the α and β chain CP domains has been conserved throughout vertebrate evolution. In contrast, the lengths of the TCR-γ and -δ chain CP domains (between the interchain Cys and the transmembrane domain) are comparable in each species examined thus far (22, 23). Therefore, a length similarity between the δ and γ chains has been conserved in this region in γδ receptors during a similar period of vertebrate evolution.

Functionally, the CP domains may mediate an interaction with the ζ homodimer. In this regard, α/β TCRs compete more avidly for the ζ chain compared to α/δ TCR (27). Furthermore, mutation of the α-CPM leads to a TCR complex that is unstably associated with the ζ chains (15). Nevertheless, why the CP domains have diverged between α/β and γ/δ TCRs is puzzling. It is conceivable that the CP domains in the α/β TCR have a specialized function such as interacting with the CD4 and CD8 coreceptors or that they have evolved to optimize the efficiency of signal transduction from MHC encoded ligands.
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