THE LYSINE RESIDUE IN THE MEMBRANE-SPANNING DOMAIN OF THE β CHAIN IS NECESSARY FOR CELL SURFACE EXPRESSION OF THE T CELL ANTIGEN RECEPTOR

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The TCR recognizes antigens associated with MHC molecules (1). This recognition event is then transduced across the plasma membrane to initiate intracellular biochemical events that include phosphatidylinositol hydrolysis and tyrosine kinase activation (2). The remarkable complexity of the TCR is likely to have evolved to carry out efficiently both its recognition and its signal tranduction functions.

The TCR consists of a molecular complex of at least seven integral membrane protein chains (Fig. 1) (1-4). On most T cells, the TCR contains a clonally distributed disulfide-linked heterodimer (Ti) of an α and a β chain, which together are sufficient for the recognition of both antigen and the restricting MHC molecule (5, 6). A distinct form of Ti consisting of a γ/δ chain heterodimer is present on a subpopulation of T cells but its function is unknown (7, 8).

Associated with both forms of Ti are at least five invariant peptide chains composing the CD3 complex, the CD3-δ, -ε, -γ, and disulfide-linked ζ chains (2-4). Some TCR complexes may contain a ζ chain that is disulfide-linked to another protein referred to as the CD3 η chain (9). The short cytoplasmic tails of Ti chains (5-12 residues) compared with the extensive cytoplasmic domains of the CD3 chains (40-113 residues) (1-4, 10), the agonist properties of CD3 mAbs (reviewed in references 2-4), and studies with somatic cell mutants (11), suggest that CD3 plays a role in signal transduction.

The nature of the structural and functional association between the Ti and CD3 subunits is of considerable interest. Cointernalization (12), coimmunoprecipitation (13), and crosslinking studies (14) have demonstrated their association on the cell surface. Studies with somatic cell mutants have established that the cell surface expression of CD3 is dependent upon the Ti heterodimer (15). Individual chains of CD3 can also limit cell surface expression of the entire TCR complex as evidenced in studies with CD3 ζ chain–deficient cells (16). The variable influence of detergent

concentration upon preservation of CD3 and Ti association suggests that hydrophobic protein domains and/or protein-phospholipid interactions may be important in the CD3-Ti association (17). One striking structural feature of the deduced primary sequence of all of the component Ti and CD3 chains that may relate to their structural and functional association is the unusual conservation of charged residues within their transmembrane domains (2-4, 10). Basic lysine and arginine residues are present in all four of the Ti chains (α, β, γ, and δ), and acidic glutamic or aspartic residues are present in the CD3-δ, -ε, -γ, and -ζ chains (Fig. 1). It has been proposed that these unusually placed oppositely charged residues are responsible for the CD3-Ti association, perhaps through salt bridges. The transmembrane lysine residue in the Ti-β chain is conserved in human (18), murine (19), feline (20), and rabbit species (21). Using a β chain–deficient mutant of the human T cell line Jurkat, we used site-directed mutagenesis to examine the importance of this residue.

Materials and Methods

Cells.   The human T cell line Jurkat and a mutant of Jurkat, J.RT3-T3.5, which fails to express the TCR complex due to deficient full-length Ti β chain transcription, were previously described (15).

Mutagenesis.   A 2.2-kb Xbal-Bam HI fragment from pTβF-neo (15), containing the wild-type Ti β chain cDNA, was ligated into M13mp10. Site-directed mutagenesis was carried out using the double primer approach (22). All mutant clones were then sequenced by the dideoxynucleotide method (23) to ensure that no other changes had been introduced. Once a mutant containing the desired base change was isolated, the replicative form of the M13mp10 was isolated using the alkaline-lysis method for plasmid preparation (23) and the 2.2-kb Xbal-Bam HI fragment was re-excised . A pTβF-neo derivative, pTglob, was prepared by substituting a 600-bp β globin fragment for the 2.2-kb Ti β chain cDNA. The mutant Ti β chain cDNA fragments were then religated into this vector. Thus, any recircularization of the vector with its own insert could be readily distinguished from plasmids containing mutant Ti β chain cDNA inserts.

Transfections.   J.RT3-T3.5 cells were transfected with plasmid DNA by protoplast fusion or electroporation as previously described (11, 15). Transformants were selected for by resistance to geneticin (2 mg/ml; Sigma Chemical Co., St. Louis, MO).

RNA Analyses.   Geneticin-resistant clones were screened by RNA dot-blot analysis and Northern blot analysis using the full-length β chain cDNA (15), the Xbal-Pvu II fragment of the Jurkat Vβ cDNA, or the full-length Jurkat α chain cDNA (24).

Briefly, cytoplasmic extracts of cells were prepared by lysing cells in a solution containing 0.5% NP-40 in 10 mM Tris (pH 7.0), 1 mM EDTA and sedimenting the particulate fraction. 50 μl of supernatant derived from 1-2 × 10^6 cells were diluted with 30 μl of 20× SSC (3 M NaCl, 0.3 M trisodium citrate, pH 7.0) and 20 μl of 37% formaldehyde. The mixture was heated at 60°C for 15 min and either frozen or used immediately in a dot-blot manifold apparatus (Hoefer Scientific Instruments, San Francisco, CA). Serial dilutions of cell extracts were aspirated onto nylon filters and hybridized as previously described. Geneticin-resistant clones hybridizing with the Jurkat Vβ cDNA probe were further analyzed by Northern blot analysis.

RNA for Nothern blot analyses was isolated by either the guanidinium thiocyanate method (23) or by the following method for large numbers of samples. Cells were lysed by vortexing in lysis buffer (0.65% NP-40; 10 mM Tris, pH 7.8; 1.5 mM $MgCl_2$; 150 mM NaCl). Nuclei and large cell fragments were pelleted by low-speed centrifugation and the resulting supernatant was treated with an equal volume of 7 M urea; 1% SDS; 10 mM Tris, pH 7.5; 10 mM EDTA; 350 mM NaCl, and incubated for 10 min on ice. The solution was then extracted with phenol and RNA was precipitated with 2.5 vol of ethanol. Northern blots were prepared and hybridized as previously described (15).

Immunofluorescence and Flow Cytometry.   Cells were stained by indirect immunofluorescence

with the indicated mAb as previously described (11, 17). Cell surface immunofluorescence was assessed by flow cytometry using the FACScan (Becton Dickinson & Co., Mountain View, CA).

Protein Sequence Analysis. The Chou-Fassman algorithm (25) to predict secondary protein structure was performed using software available from Intelligenetics, Inc. (Mountain View, CA).

Results and Discussion

Conservation of oppositely charged amino acid residues within the transmembrane domains of the chains of the Ti heterodimers and CD3 complex (Fig. 1) has been observed in all of the chains of the complex that have been cloned to date (reviewed in references 2–4). To assess the function of these unusually placed acidic and basic residues, site-directed mutagenesis was performed of the lysine residue within the Ti β chain transmembrane domain, lysine[290] (26).

We utilized as the recipient cell in these transfection experiments a mutant of the human T cell line Jurkat, J.RT3-T3.5, that fails to express substantial levels of cell surface CD3 or the Ti heterodimer due to deficient expression of full-length 1.3-kb Ti β chain transcripts (15). J.RT3-T3.5 still transcribes the nonfunctionally rearranged 1.0-kb transcript, resulting from a D-J rearrangement only, derived from the other allele (18). High level expression of both CD3 and the Ti heterodimer can be restored by expression of a wild-type Ti β chain cDNA that is constitutively transcribed under the influence of the Friend spleen focus-forming virus long-terminal repeat in pTβF-neo (15).

The Ti β chain lysine[290] was mutagenized to arginine, glutamic acid, glutamine, serine, or leucine. J.RT3-T3.5 was transfected by protoplast fusion or electroporation with the pTβF-neo containing the wild-type Ti β chain sequence or the mutants at position 290. These plasmids also contain the neomycin resistance gene. At least 12 independent geneticin-resistant clones of each plasmid transfection were screened by RNA dot-blot or Northern analyses. In each transfection, at least 50% of geneticin-resistant cells expressed a new 3.4-kb Ti β chain transcript. Whereas all cells contained the 1.0-kb Ti β chain transcript, only transfected cells contained the 3.4-kb transcript, which hybridizes with the Vβ cDNA probe (unlike the 1.0-kb transcripts, which lack V region sequence [18]) and serves as a convenient marker for transcripts derived from the transfected cDNA. The larger transcript size results from the use
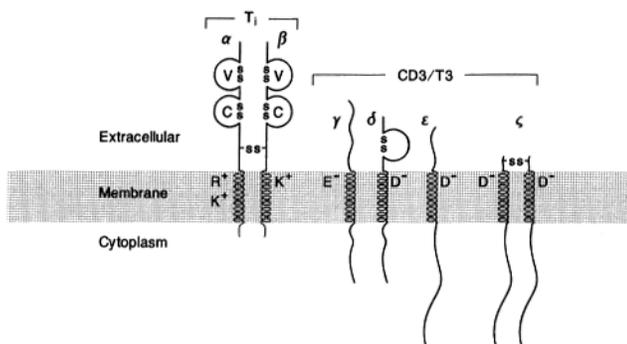
FIGURE 1. Schematic model of the human T cell antigen letters. The single letter code for amino acids was used to designate charged residues within the membrane-spanning domains.

of a downstream polyadenylation addition site in the expression vector (15). A Northern blot analysis of representative clones is depicted in Fig. 2.

All clones expressing the 3.4-kb transcript were then analyzed for cell surface receptor expression by immunofluorescence and flow cytometry using the following mAbs: anti-Leu-4 (anti-CD3); WT31 (anti-Ti$_{\alpha/\beta}$); and C305 (anti-Ti, Jurkat β chain specific). A consistent phenotype was observed using all three antibodies with all transfected cells. Examples of these findings obtained with anti-Leu-4 and WT31 are presented in Fig. 3 using the same clones of cells depicted in Fig. 2 that expressed abundant levels of the 3.4-kb transcript. High level cell surface expression of both the Ti heterodimer and CD3 could only be restored by the construct containing
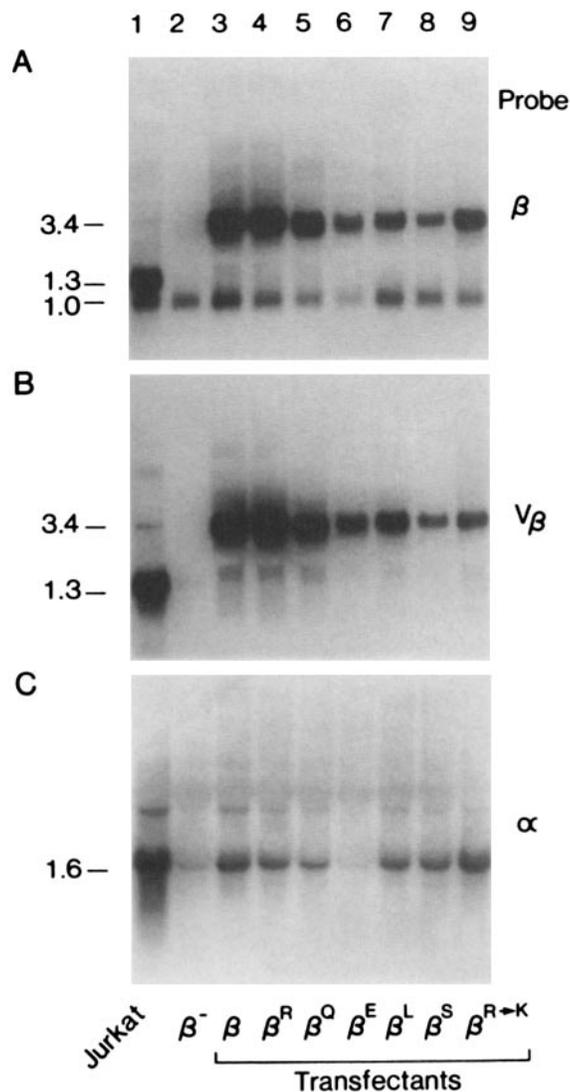

Figure 2.   Northern blots of RNA isolated from Jurkat cells (lane 1), β chain–deficient mutant J.RT3-T3.5, which served as the recipient in transfections (lane 2), and transfectants (lanes 3-9). A single letter superscript is used to designate the mutations at position 290 from the wild-type lysine residue. The transfectant in lane 9 represents a cell transfected with cDNA in which the arginine[290] mutant cDNA served as a template to back-mutate that residue to lysine using the AAA codon. Blots were hybridized with a full-length Ti β chain cDNA probe (A), a Vβ-specific cDNA probe (B) or a full-length Ti α chain probe (C).
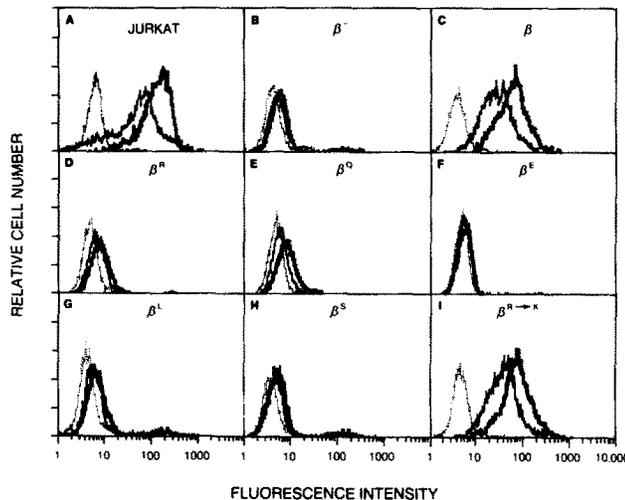
FIGURE 3. Flow cytometry histograms of Jurkat (*A*), β chain-deficient mutant J.RT3-T3.5 (β⁻; *B*), which served as the recipient in transfections, and transfectants (*C–I*) stained with the following mAbs: IgG control (*dotted lines*); WT31, anti-Ti α/β (*thin solid lines*); or anti-Leu-4, anti-CD3 (*thick solid lines*). A single letter superscript is used to denote the mutation at lysine[290].

the wild-type sequence (Fig. 3 *C*), i.e., lysine at residue 290. In contrast, cells expressing arginine, glutamine, glutamic acid, leucine, or serine residues at position 290 failed to express appreciable levels of the TCR on the cell surface as assessed by immunofluorescence with any of the three mAbs used that react with distinct antigenic determinants of CD3 or Ti (Fig. 3, *D–H*). The use of three mAbs that react with distinct determinants of the CD3/Ti complex argues that the failure to detect the CD3 or Ti on the cell surface was not due to TCR conformational changes induced by the mutated transmembrane residue.

Levels of 3.4-kb transcripts varied among clones but failure of expression of cell surface of CD3 and Ti could not be attributed to these differences since lower levels of transcripts derived from wild-type cDNA transfectants still restored abundant levels of CD3 and Ti on the cell surface (data not shown). To rule out the possibility of errors in mutagenesis, such as second site mutations, the cDNA construct containing the arginine[290] mutation was used as a template. Arginine[290] was back-mutated to lysine using the alternate codon for lysine, AAA, rather than AAG contained in the wild-type cDNA. This construct did reconstitute cell surface expression of CD3 and Ti determinants (Fig. 3 *I*).

These results strongly argue that the lysine residue at position 290 within the Ti β chain transmembrane domain is critical for TCR expression. This is consistent with the observed conservation of lysine at this position in human, murine, rabbit, and feline Ti β chains (18–21). It has been proposed that the basic residue at this position may be important in the association with CD3 chains, which all contain acidic residues in their transmembrane domains. The failure of the basic arginine residue to substitute for lysine suggests that side chain structure, as well as charge, may be an important determinant at this residue. Since the structure of the transmembrane domain of the Ti β chain is not known, we used a Chou-Fassman algorithm (25) to predict the secondary structure of this domain. The value of such predictions for protein transmembrane domains is not known. Nevertheless, at lysine[290] in the wild-type β chain, a transition from β-pleated sheet to α helix is

predicted to occur. At this position the algorithm predicts that glutamic acid, but not arginine, preserves this transition in structure. However, the failure of glutamic acid to reconstitute CD3/Ti expression suggests that secondary structure may not be the only important feature. Since such charged residues are unusual in transmembrane domains, we also mutated this residue to serine and leucine, residues commonly expressed within transmembrane domains. These mutant cDNAs, likewise, failed to restore CD3/Ti expression. According to the Chou-Fassman algorithm (25), these changes would have altered the predicted transition in secondary structure occurring in the wild-type protein at position 290.

The dynamic assembly of the CD3/Ti has received considerable recent attention (4). The failure of the arginine or other substitutions to reconstitute CD3/Ti could be due to the instability of the mutant β chain proteins. However, biosynthetic labeling studies of the cells transfected with the Ti β chain cDNA containing the arginine mutation suggest that the Ti β chain protein is synthesized at levels comparable with those observed with the wild-type cDNA (data not shown). Association between the CD3 complex and the arginine[290] mutant Ti β chain protein has not been observed in preliminary biosynthetic labeling studies. This suggests that lysine at position 290 may be important at an early step in the assembly of the CD3/Ti complex.

The TCR represents one of the most complex plasma membrane receptors described to date. The Ti structure appears to function primarily as a ligand-binding subunit, and the associated CD3 complex is thought to be involved in a signal-transducing function perhaps by coupling the occupied Ti subunit to the intracellular signal transduction mechanism. Indeed two distinct alternative ligand binding subunits, Ti α/β and Ti γ/δ, are coupled to CD3. A striking structural feature of the component chains is the conserved transmembrane charged residues. This study would suggest that, at least in the case of the Ti β chain, conservation of structural features of these transmembrane residues, in addition to charge properties, are important.

The function of these charged residues has been attributed to the structural association of the Ti subunit to CD3. In view of the relatively rigid constraints for conservation of the lysine within the Ti β chain membrane-spanning domain, one may speculate upon additional functions of these transmembrane domains. The transfer of information across the plasma membrane to signal the cytoplasmic domain of receptor occupancy is an issue of considerable importance. One possible mechanism to accomplish such information transfer is to change conformationally the relationship between interacting charged amino acids within the plasma membrane. This function would likely impose constraints upon the structural features of the transmembrane domains to allow appropriate chain interactions. This would be consistent with the observed conservation across species for the lysine residue of the Ti β chain at position 290. In this regard, it is noteworthy that other physiologically active receptors with multiple transmembrane-spanning domains also contain charged residues. The family of receptors homologous with the β2 adrenergic receptor, which interact with GTP-binding proteins, have seven transmembrane spanning domains, some with conserved charged residues like the TCR, albeit the seven membrane-spanning domains are contained within a single polypeptide chain (27).

Hence, these receptors may utilize a common mechanism for transducing a state of receptor occupancy to cytoplasmic domains to initiate intracellular responses.

Summary

The TCR is a complex receptor composed of seven polypeptide chains consisting of a ligand-binding subunit, Ti, and a putative signal-transducing subunit, CD3. Phylogenetically conserved charged amino acid residues within the membrane-spanning domains present in all seven chains of the TCR have been proposed to be important in the association between Ti and CD3. Using a Ti β chain–deficient mutant of the cell line Jurkat, site-directed mutagenesis and transfection of Ti β chain cDNA was performed to assess the importance of the lysine residue at position 290 within the membrane-spanning domain of the Ti β chain to expression of the TCR complex. These studies demonstrated that the lysine residue, and not simply conservation of either basic charge or secondary structure, is important at this position.

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