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25. The EVF can be shown algebraically to be a function of the mean size of objects (cells and processes) occupying the space and the mean distance between objects. If the objects occupying extracellular space are modeled as cubes of width w, EVF = $\Sigma A_n[(w_n + d)^3 - w_n^3]/(w_n + d)^3$, where n is the number of different-sized objects, A_n is the fraction of each object ($\Sigma A_n = 1$), and d is the mean interobject distance. Given fixed d = 30 nm, if the space were occupied only by cubes of width 450 nm (for example, processes), EVF = 0.176; however, with cubes of 10-μm width (cell bodies), EVF = 0.009. With an equal number of large and small cubes, EVF = 0.102. The essential point is that the smaller the mean size of objects, the larger the expected EVF. Further tests of this analysis would require quantitative measurements from electron micrographs of the different hippocampal regions.
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Expression of T Cell Antigen Receptor Heterodimers in a Lipid-Linked Form

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The interaction of the T cell receptor for antigen (TCR) with its antigen–major histocompatibility complex ligand is difficult to study because both are cell surface multimers. The TCR consists of two chains (α and β) that are complexed to the five or more nonpolymorphic CD3 polypeptides. A soluble form of the TCR was engineered by replacing the carboxyl termini of α and β with signal sequences from lipid-linked proteins, making them susceptible to enzymatic cleavage. In this manner, TCR heterodimers can be expressed independently of the CD3 polypeptides and in significant quantities (0.5 milligram per week). This technique seems generalizable to biochemical and structural studies of many other cell surface molecules as well.

THE ANTIGEN-SPECIFIC IMMUNE RESPONSES of vertebrates are determined by B and T lymphocytes. B cell specificity is due to immunoglobulin molecules that can be either expressed on the cell surface or secreted. Extensive information has been obtained regarding the biochemistry and structure of immunoglobulins and their interactions with various antigens (*1, 2*). In contrast, little is known about the structure of TCR heterodimers or their interaction with their putative ligands, antigen fragments embedded in molecules of the major histocompatibility complex (MHC) (*3*). This is in part because the TCR is not secreted, and it is not expressed on the surface of T cells in large amounts [~20,000 to 40,000 molecules per cell (*4*)]. The complexity of the TCR ligand and its normal cell surface expression has also contributed to the difficulties in trying to characterize the mode of interaction. In addition, although the TCR polypeptides are immunoglobulin-like (*5*), they are always co-expressed with CD3 molecules (*6*), which leaves open the possibility that they require those molecules for their stability. To better understand TCR-mediated recognition, we and others have unsuccessfully tried to engineer TCR α and β polypeptides, or variable (V) regions derived from them, as antibody chimeras (7) or truncated molecules (*8*), to allow the expression of a soluble form in biochemically significant quantities.

The COOH-terminal 37 amino acids of decay-accelerating factor (DAF) can serve as a signal sequence for the attachment of an otherwise secreted protein to the cell surface by means of a phosphotidyl inositol (PI)–glycan linkage (*9*), which can be cleaved off the surface of transfected cells by the specific enzyme, phosphatidyl inositol–specific phospholipase C (PI-PLC) (*10*). Expression of a recombinant TCR heterodimer anchored by a PI-glycan linkage would provide: (i) a PI-PLC–cleavable, soluble form without a need for detergents; (ii) TCR surface expression that is not limited by the amount of CD3 (*11*); and (iii) a lipid-linkage that should keep the TCR polypeptides in the correct orientation with respect to each other and at a relatively high concentration (in the plane of the interior membrane), thus maximizing the chances of correct association.

The T helper hybridoma 2B4 (*12, 13*) bears an αβ TCR that recognizes a fragment of pigeon cytochrome c plus the class II MHC molecule, I-E^k (*14*). We replaced the transmembrane and cytoplasmic domains of both chains with either the signal sequence from a monomeric protein, DAF (*8*), or with COOH-termini derived from human placental alkaline phosphatase (HPAP) (*15*), a PI-linked dimer. Because the sequences required for HPAP to direct PI-linkage have not been defined, we designed two HPAP signals of different lengths that contained either the last 38 (HPAP-S) or the last 47 amino acids (HPAP-L) of the protein (Fig. 1A). By analogy with immunoglobulin Fab fragments, all three PI-anchoring signal sequences were joined to the TCR chains at the fifth residue COOH-terminal of the last conserved cysteine. The modified α and β cDNAs were then inserted into the mammalian expression vector pBJ1-Neo (*16*). Staining of COS cell transfectants with monoclonal antibodies specific for the α [A2B4.2, (*13*)] and the β [KJ25, anti-$V_\beta 3$ (*17*)] TCR chains revealed the presence of both on the cell surface. Ninety percent of the TCR molecules could be cleaved from the membrane after treatment of the cells with PI-PLC from *Bacillus thuringiensis* (*10*), thus all the COOH-termini used here could serve as signal sequences for this type of linkage. The same constructs were then introduced into Chinese hamster ovary (CHO) cells, and stable transfectants resistant to the antibiotic, G418, were selected.

In order to determine the stability of expression of the PI-anchored TCR on the cell surface, the brightest 5% αβ-staining cells of each pool of CHO transfectants (αβDAF, αβHPAP-S, and αβHPAP-L) were pooled after fluorescence-activated cell sorting. The same procedure was repeated on the sorted cells after 2 weeks of culture. The staining profiles obtained (Fig. 1B) after the second sorting of each CHO transfectant pool were obtained with subsaturating amounts of the antibody to V_α, A2B4.2, so that it would not block the binding of the antibody to $V_\beta 3$, KJ25 [(*18*) and below]. At this point, αβHPAP-S cells were a homogeneous population of bright double-positive cells, whereas both the αβDAF and αβHPAP-L cells contained significant numbers of dully staining cells (Fig. 1B). This suggests that the HPAP sequences, especial-

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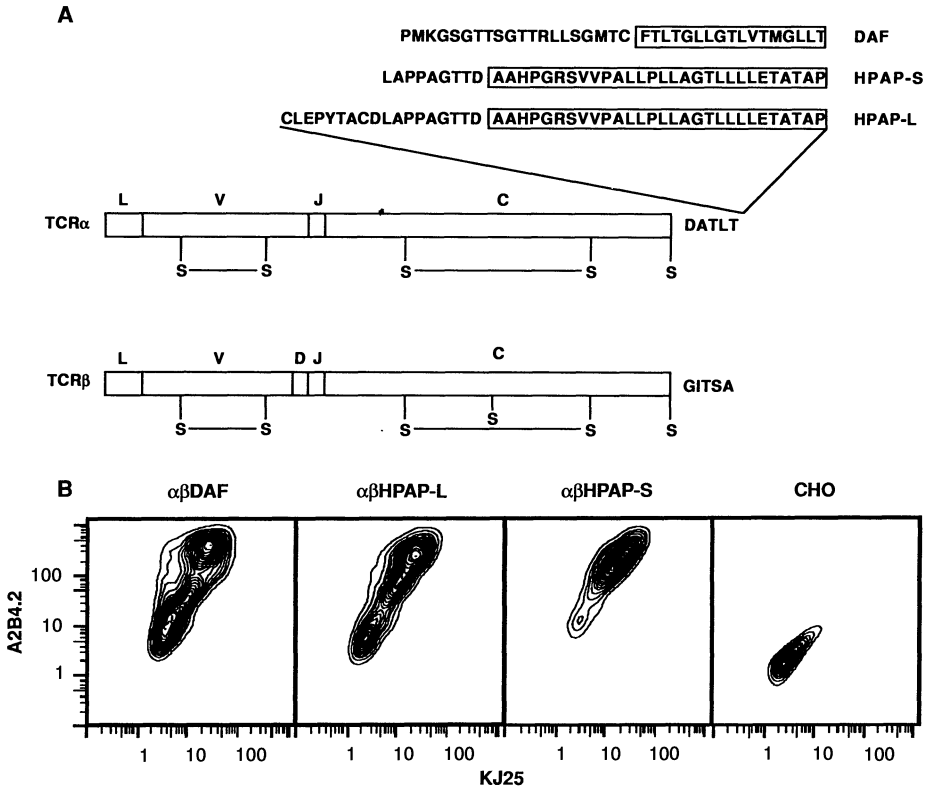

Fig. 1. (**A**) Diagram of TCR α and β chains with DAF and HPAP PI-anchoring signals. The PI-anchored TCRs were constructed by fusing the PI-anchor signals (DAF or HPAP) to the fifth amino acid residue of the TCR located 3′ to the last cysteine residue before the transmembrane domain. Full-length cDNAs encoding the 2B4 TCR α or β chains (*24, 25*) were inserted 5′ to the DNA segment coding for the last 37 amino acids of DAF (residues 311 to 347) (*26*) or the last 38 or 47 amino acids of HPAP (residues 476 to 513 and 467 to 513, respectively) (*15*) cloned in plasmid Bluescript pSK$^-$ (Stratagene). The in-frame joining between the TCR and the PI-anchor signal was performed by oligonucleotide-directed in vitro deletional mutagenesis (*27*). After mutagenesis, the first 227 and 265 amino acid residues of the TCR α and β chains, respectively, were retained in the chimeric fusion protein. The truncated α and β chains were fused to residue 311 of DAF or to residue 484 (HPAP-L) or 495 (HPAP-S) of HPAP. (**B**) Comparison of CHO transfectants coexpressing TCR α and β chains on the cell surface with various PI-anchoring signals. CHO cells were transfected with αβDAF, αβ-HPAP-L, or αβHPAP-S constructs [inserted into pBJ1-Neo (*16*)] by electroporation (*28*). The G418-resistant colonies were pooled and analyzed for surface expression of the PI-anchored TCR α and β chains. Cells were sequentially stained at 4°C for 1 hour in phenol red–free RPMI plus 5% fetal bovine serum with KJ25 (anti-$V_\beta 3$) and biotinylated A2B4.2 (antibody to V_α). Fluorescein isothiocyanate (FITC)–conjugated goat antibodies to hamster immunoglobulins and streptavidin-PE were used as secondary antibodies.

ly HPAP-S, may be superior to DAF in expressing multimeric chimeras. Because of this greater apparent stability, we have used the αβHPAP-S transfected cells for all subsequent studies.

To demonstrate that heterodimers between α and β chains were being formed juxtaposing V_α and V_β determinants, we made use of the inhibitory effect that incubation of 2B4 TCR-bearing cells with saturating amounts of A2B4.2 antibody has on the binding of the KJ25 antibody (*18*). When 2B4/Jurkat cells [a human T cell tumor line transfected with 2B4 TCR α and β chain genomic constructs (*19*)] are first treated with the antibody to V_α and then with the anti-$V_\beta 3$, staining with the latter is completely inhibited (compare A and B of Fig. 2). The same inhibition effect can be observed after pretreatment of αβHPAP-S (Fig. 2, C and D), αβDAF, and αβHPAP-L (*20*) transfectants. KJ25 staining of the CHO cells is inhibited by 90% after preincubation with A2B4.2, thus, most of the PI-anchored TCR β chains were expressed on the cell surface as heterodimers with the α chain and in a conformation indistinguishable from the native structure. This is despite the absence of CD3 expression in this type of cell.

To study the biochemical characteristics of the TCR, we purified PI-PLC–cleaved TCR heterodimers to near homogeneity. The CHO αβHPAP-S cells were subcloned

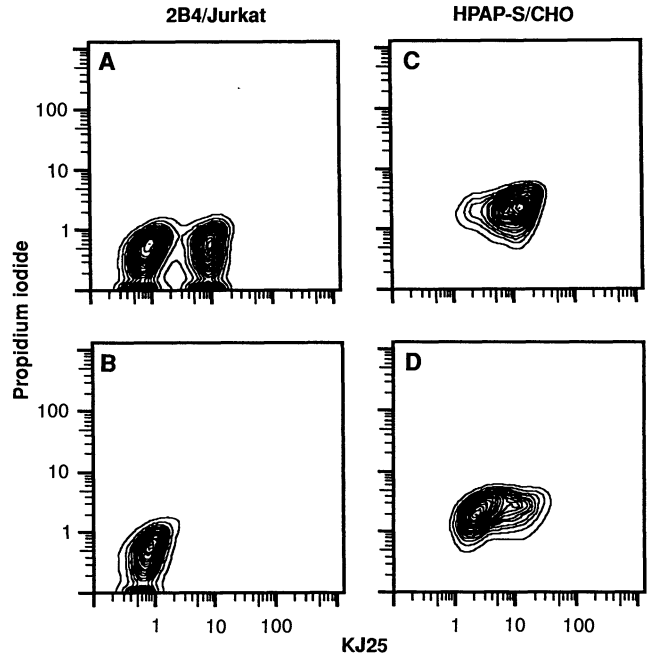

Fig. 2. Association of V_α and V_β demonstrated by cross-blocking antibodies. Jurkat β$^-$ cells (5×10^5) expressing a functional 2B4 αβ TCR after transfection with 2B4 α and β genes (*14, 19*) (**A** and **B**) and CHO cells transfected with the αβHPAP-S construct (**C** and **D**) were pretreated with (B and D) or without (A and C) A2B4.2 (20 μg/ml) and then stained with KJ25 (2 μg/ml) and FITC-conjugated goat antibodies to hamster immunoglobulins (A2B4.2 is a mouse antibody and KJ25 is from a hamster such that only the latter will stain with the FITC reagent). Cells were analyzed by flow cytometry after staining. The data are plotted as log fluorescent intensity in arbitrary units. Approximately half of the 2B4 αβ transfected Jurkat cells are negative for TCR, and half are positive, accounting for the two populations visible in the profile. The *y*-axis shows propidium iodide staining, a measure of cell viability, while the *x*-axis shows the degree of KJ25 fluorescence.

and a clone that was a high expresser of αβ was grown to about 2×10^{10} cells in a hollow fiber bioreactor. The cells were incubated every 2 days with ~10 units of purified PI-PLC (*10*), and the culture supernatant was collected. The TCR proteins were purified by sequential affinity column chromatography. The first column used for purification was the antibody to V_α coupled to Sepharose. Analysis of eluate on a polyacrylamide-SDS gel (Fig. 3) under nonreducing conditions revealed the presence of high molecular weight dimers (70 kD) (αβ heterodimers as well as α homodimers) and some

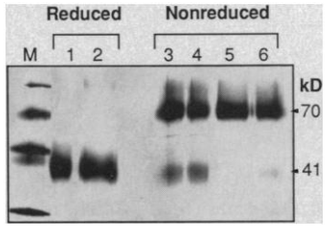

Fig. 3. Purification of soluble TCR heterodimer and gel analysis. αβHPAP-S transfected CHO cells (2×10^8) were seeded into a Cell Pharm I Bioreactor (CD Medical). After 2 weeks of culture (2×10^{10} cells present in the Bioreactor), the cells were treated with ~10 units of PI-PLC at 37°C for 3 hours (*25*). The chimeric TCR was recovered in 250 ml of growth media pulsed into the Bioreactor. The supernatant was filtered, adjusted to pH 8.0, and passed over an A2B4.2 (cyanogen bromide–coupled Sepharose) affinity column (1 ml) preequilibrated with 0.1 M phosphate and 0.15 M NaCl, pH 8.0. The column was then washed with the same buffer. The protein was eluted with 0.1 M NaOAc and 0.15 M NaCl, pH 3.5, buffer in 0.5-ml fractions and neutralized immediately with 2 M tris-HCl, pH 8.5 (1/10 volume added). The first four fractions, which contain most of the soluble TCR, were pooled and applied to a KJ25 affinity column. The protein was then eluted at pH 5.0 with 0.1 M NaOAc and 1 M NaCl. Sample eluates from the A2B4.2 (lanes 1, 3, and 4) or A2B4.2 + KJ25 (lanes 2, 5, and 6) columns were separated by electrophoresis on a 10% SDS–polyacrylamide gel under reducing (lanes 1 and 2) and nonreducing (lanes 3 to 6) conditions. When run under nonreducing conditions, the samples were either not boiled (lanes 3 and 5) or boiled (lanes 4 and 6) before being loaded on the gel. The gel was stained with silver nitrate (*29*). The fraction eluted from the A2B4.2 column, not boiled (lane 3) or boiled (lane 4) before being loaded on the gel, contains both monomers (α, 41 kD) and dimers (αα and αβ, 70 kD). After subsequent passage of this fraction over the KJ25 column and elution at pH 5.0, only dimers are detectable (lane 5, sample not boiled) showing a separation of αβ heterodimers from α monomers and αα dimers. If the sample is boiled (lane 6), low quantities of monomers become detectable. Under reducing conditions (lanes 1 and 2), dimers are reduced to the size of monomers.

α monomers (41 kD). Both 2B4 α and β chains have the same molecular weight (41 kD) and therefore cannot be distinguished from each other on the gel. The amount of monomer was slightly increased when the sample was boiled, indicating that a small percentage of the dimeric molecules are not disulfide-linked. Passage of the fraction eluted from the A2B4.2 column over the anti-$V_\beta 3$ (KJ25) column allowed separation of αβ heterodimers from α homodimers and α monomers. Again, boiling of the sample results in the appearance of a small amount of monomer showing that most (but not all) of the heterodimeric molecules are disulfide-linked. The fact that the non–disulfide-linked heterodimer is dissociated by boiling, yet not by the presence of SDS alone (at room temperature), may be further evidence of a strong association of the TCR α and β chains, as previously noted for class II MHC molecules (*21*). From the result of the silver-stained gel, we estimate that the TCR molecules cleaved off the surface of CHO cells are composed of 40% monomers and 60% dimers among which 50% are heterodimers (30% of the total). We can obtain ~0.5 mg of TCR heterodimer per week, making possible a variety of biochemical and structural studies. Binding of labeled soluble TCR to peptide-pulsed I-E^k bearing cells has been attempted, thus far without success (*22*). This may be because the affinity of T cell receptors for their ligands is very low or that the number of peptide MHC complexes is small, or a combination of these two factors.

In summary, we have extended the use of PI-linkage signal sequence chimeras to include a TCR αβ heterodimer. Even in the absence of CD3 molecules, this heterodimer has all of the characteristics of the native structure that we have been able to assay for. This should be particularly important for the study of T cell recognition because so much of what is thought to be true is inferential and cannot presently be assayed directly. Furthermore, this strategy seems generally applicable to other cell surface multimers, as indicated by its recent application to class II MHC heterodimers (*23*).

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