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34. A more elaborate discussion of this model, including a proposed mechanism of rotamase catalysis, is presented elsewhere (*3*).
35. Although the affinity of FK506 for FKBP59 is less than it is for FKBP12 (K_d = 0.4 nM), the relevant affinity may be that of the immunophilin-drug complex for its target, which is analogous to the way immunosuppressants block signal transduction pathways (*3*). For example, despite having a 100-fold lower affinity for cyclophilin than cyclosporin A, $MeBm_2t^1$-CsA potently inhibits T cell receptor–mediated signal transduction because its cyclophilin complex is a high-affinity ligand to calcineurin (J. Liu *et al.*, *Biochemistry* **31**, 3896 (1992).
36. Okadaic acid (OA), a potent inhibitor of the serine-threonine protein phosphatases PP2A and PP1, activates the progesterone and estrogen receptors in a ligand-independent manner [R. F. Power, S. K. Mani, J. Codina, O. M. Conneely, B. W. O'Malley, *Science* **254**, 1636 (1991)]. OA potentiates the ability of dopamine, acting through the D_1 receptor, to activate these receptors as well. However, neither OA nor dopamine activates the human GR. By a similar mechanism that involves inhibition of calcineurin (*2*) or a related phosphatase, the FKBP59-FK506 complex may activate GR in a ligand-independent manner or potentiate ligand-independent activation by membrane-associated events.
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High-Efficiency Expression and Solubilization of Functional T Cell Antigen Receptor Heterodimers

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The T cell receptor (TCR) ζ chain was attached to the TCR α and β extracellular domains to induce efficient expression of αβ heterodimers that can recognize complexes of antigen with major histocompatibility complex (MHC) molecules. Chimeric constructs expressed in RBL-2H3 cells were efficiently transported to the cell surface uniquely as disulfide-linked heterodimers. Transfectants were activated by specific antigen-MHC complexes, which demonstrated that the expressed αβ was functional and that CD3 was not required for antigen-MHC binding. Constructs with thrombin cleavage sites were efficiently cleaved to soluble disulfide-linked heterodimers. Thus, attachment of TCR ζ domains and protease cleavage sites to TCR α and β induces expression of demonstrably functional heterodimers that can be solubilized.

Determination of the three-dimensional structure of the TCR αβ heterodimer requires the production of large amounts of a soluble form of this complex. However, isolation of the soluble αβ heterodimer presents many difficulties. The α and β chains are both type 1 transmembrane proteins (*1*) and as such are not soluble in the absence of detergent. Furthermore, the αβ heterodimer is not expressed at the cell surface unless associated with the other chains of the TCR complex (*2*). In addition, structural alterations to circumvent the problems of solubility and expression efficiency should not affect the heterodimer combining site. Although soluble forms of the TCR αβ heterodimer have been produced (*3*), it is not clear whether they can bind a specific antigen-MHC complex.

We attached the transmembrane and cytoplasmic domains of the TCR ζ chain to the extracellular domains of the α and β chains to facilitate production of the αβ heterodimer. The cytoplasmic domain of ζ induces cell activation when attached to heterologous proteins that are cross-linked by antibody (*4*, *5*). Thus, the ability of an α-ζ/β-ζ heterodimer to bind antigen-MHC could be tested by exposure of the cells that expressed this heterodimer to antigen-pulsed presenting cells. In addition, the ζ transmembrane domain induces disulfide dimerization when attached to heterologous proteins (*4*). Because cells that express glycosyl-phosphatidylinositol–linked α and β chains have more than 99% of these constructs retained in the endoplasmic reticulum (ER) in a nondisulfide-linked form (*6*), we reasoned that inducing disulfide dimerization could enhance heterodimer expression.

Chimeric constructs made between TCR α and β cDNAs isolated from the T cell hybridoma 2B4 and the murine TCR ζ cDNA (*7*) were stably transfected into the rat basophilic leukemia line RBL-2H3 (*8*). Flow cytofluorometric analysis of stable transfectants revealed that large amounts of the chimeric constructs at the cell surface were obtained only upon expression of both constructs. The mean fluorescence of uncloned transfected cells that expressed both α-ζ and β-ζ constructs after staining with the monoclonal antibody A2B4 (anti-2B4 α chain) (*9*) was substantially greater than that of cells that expressed only an α-ζ construct (Fig. 1A). Clones have been

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obtained from lines transfected with α-ζ and β-ζ constructs that stably express surface A2B4 immunofluorescence levels as much as 200-fold over background fluorescence. In comparison, the A2B4 immunofluorescence of the 2B4 hybridoma, which expresses 20,000 to 40,000 TCRs per cell (*9*), is typically 10- to 20-fold greater than background immunofluorescence (*10*). The amount of surface A2B4 epitope expression depended on the design of the chimeric construct; most significantly, deletion of the region between the membrane proximal cysteine residue and the transmembrane domain of the α and β chains resulted in the greatest amount of cell surface expression.

To test whether the ζ chain domains enhance cell surface expression, we established lines that expressed the native 2B4α and β chains or lines that expressed constructs in which the charged residues in the α and β transmembrane domains were replaced by leucines in order to delete ER degradation and retention determinants (*11*). We compared the flow cytofluorometric profiles after A2B4 staining of lines transfected on the same day with either the neutral transmembrane 2B4α and β constructs or with α-ζ and β-ζ constructs (Fig. 1B). The small amount of A2B4 immunofluorescence observed in the neutral transmembrane line and in native αβ lines (*10*) demonstrates that the ζ chain domain is required for a large amount of surface expression of this epitope in RBL-2H3 cells.

These data suggest that efficient transport to the cell surface of α and β chains is dependent on disulfide-linked heterodimer formation induced by the ζ chain. To confirm that α-ζ and β-ζ were expressed at the cell surface as heterodimers, we subjected an A2B4 immunoprecipitate from a surface ^{125}I-labeled transfected cell line to two-dimensional nonequilibrium pH gradient electrophoresis (NEPHGE) under either reducing or nonreducing conditions, followed by SDS–polyacrylamide gel electrophoresis (PAGE) under reducing conditions. Because the isoelectric points (pI) of the α and β chains are different, an αβ heterodimer will resolve as two distinct spots when analyzed by NEPHGE–SDS-PAGE under completely reducing conditions but when subjected to NEPHGE under nonreducing conditions will appear as a single spot migrating at a pI between that of the individual α and β chains. Virtually all of the immunoprecipitated material migrated as would be predicted for a disulfide-linked αβ heterodimer (Fig. 1C). No ^{125}I-labeled β-ζ protein could be detected in the A2B4-cleared lysates, which rules out the presence of significant amounts of β-ζ homodimers at the cell surface (*10*).

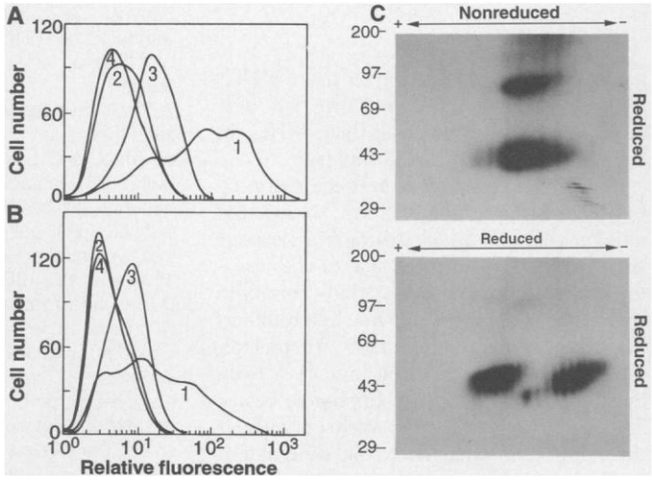

Fig. 1. (**A**) Flow cytofluorometric analysis of RBL-2H3 lines transfected on the same day with 2B4 α-ζ and β-ζ constructs or with only an α-ζ construct. Curve 1, cell line transfected with both α-ζ and β-ζ and stained with A2B4 (*9*); curve 2, same as curve 1 but stained with an isotype-matched control antibody; curve 3, cell line transfected with α-ζ construct only and stained with A2B4; curve 4, same as curve 3 but stained with control antibody. Cells were analyzed with a FACSCAN analyzer and Consort 30 program (Becton Dickinson). Constructs were made with PCR (*20*) as described (*21*), and transfection was performed as described (*18*). (**B**) Flow cytofluorometric analysis of RBL-2H3 lines transfected with 2B4 α-ζ and β-ζ constructs or with 2B4 α and β neutral transmembrane constructs. Curves 1 and 2, same as curves 1 and 2 in (A); curve 3, cell line transfected with α and β neutral transmembrane constructs and stained with A2B4; curve 4, same as curve 3 but stained with control antibody. (**C**) Two-dimensional NEPHGE–SDS-PAGE analysis of an A2B4 immunoprecipitate from a lysate of a surface ^{125}I-labeled α-ζ/β-ζ RBL-2H3 line. Samples (1.5×10^7 cell equivalents each) were run in the NEPHGE dimension under either nonreducing (top) or reducing (bottom) conditions, followed by electrophoresis on 10% SDS-PAGE gels under reducing conditions. Parallel experiments performed on untransfected RBL-2H3 cells yielded no significant signal (*10*). The band at ~95 kD in the nonreduced NEPHGE gel corresponds to incomplete reduction in the SDS-PAGE dimension. Numbers on left and corresponding dashes indicate molecular size in kilodaltons. Procedures are as described (*22*).

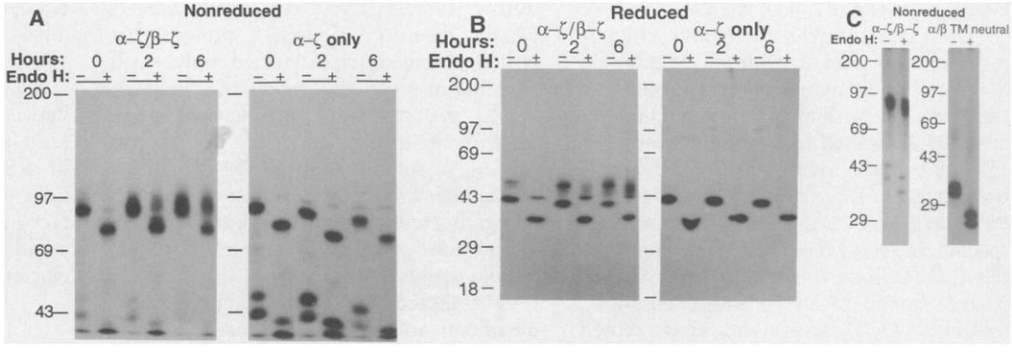

Fig. 2. Pulse-chase analysis of metabolically labeled RBL-2H3 transfectants. (**A**) Clones (3×10^7 cells each) that expressed α-ζ and β-ζ protein (left) or α-ζ protein only (right) were labeled as described (*23*). Cells were then lysed and immunoprecipitated with antiserum 386 (anti-ζ) (*12*). Equal portions of each immunoprecipitate were then treated with or without endo H as described (*24*), and half of each resulting sample wassubjected to electrophoresis on 10% SDS-PAGE gels under nonreducing conditions as described (*22*). Numbers on left and corresponding dashes indicate molecular size in kilodaltons. Incubation after pulse in the absence of labeled amino acid is indicated in hours. Parallel experiments performed on untransfected RBL-2H3 cells confirmed the specificity of the principal observed bands (*10*). Under nonreducing conditions, nondisulfide dimerized α-ζ and transmembrane neutral α chains (C) migrated at two positions. This phenomenon is not observed in T cells transfected with α-ζ constructs (*10*) and thus appears to be a result of some undefined property of RBL-2H3 cells. (**B**) Samples [as in (A)] were reduced and electrophoresed on 13% SDS-PAGE gels. (**C**) Pulse-chase endo H analysis of RBL-2H3 lines transfected with α-ζ and β-ζ constructs or transmembrane neutral 2B4α and β constructs (α/β TM neutral), showing only samples taken from the 2-hour chase point. Procedures are as described in (A) except that A2B4 was used for immunoprecipitation and the samples from the transmembrane neutral transfectant were electrophoresed on 11% SDS-PAGE gels.

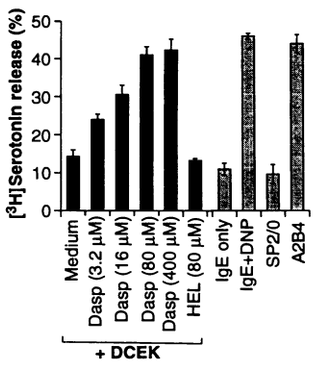

Fig. 3. Activation of an RBL-2H3 clone that expressed α-ζ and β-ζ constructs with antigen-pulsed antigen presenting cells. Also shown is the response to antibody to the 2B4 α chain and to anti-DNP IgE followed by DNP-BSA, which activates RBL-2H3 through endogenous IgE receptors (*19*). Results are calculated as counts per minute released to supernatant/(counts per minute retained by cells + counts per minute released to supernatant) and represent mean ± standard deviation of triplicate tests. The E^k-expressing fibroblast transfectant DCEK (*17*), pulsed with various concentrations of either the moth cytochrome c analog Dasp (*25*) or the control peptide HEL (*26*), was used for stimulation. Untransfected cells do not respond to antigen-pulsed DCEK cells nor do they respond to A2B4 (*10*). Procedure is as described (*27*); SP2/0 ascites were used as a control.

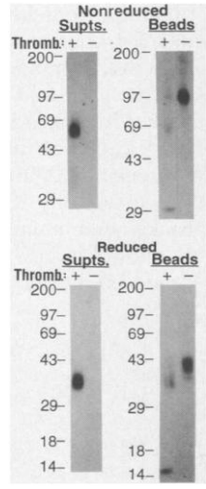

Fig. 4. Solubilization of TCR αβ heterodimers by thrombin digestion. Immunoprecipitates from a surface-iodinated α-ζ/β-ζ clone (bound to 386-coated protein A–Sepharose beads) (Pharmacia) representing 10^7 cell equivalents were resuspended in buffer that contained 100 mM tris-HCl (pH 8.5), 0.5 mM EDTA, and 3.5 mM $CaCl_2$ with or without 2 units of thrombin (Sigma) and incubated for 1 hour at 25°C. After digestion, the supernatant was recovered, and Laemmli buffer (*28*) was added to ×1 concentration; the material still bound to the beads was eluted with Laemmli buffer. Samples were then split into two equal portions and electrophoresed on 11% and 13% SDS-PAGE gels under nonreducing and reducing conditions, respectively. Numbers on left and corresponding dashes indicate molecular size in kilodaltons. Supts., supernatant samples; Beads, bead-eluted samples; Thromb., thrombin. The clone used in this experiment expressed constructs that consist of the extracellular domains of the 2B4 α and β chains terminating at the membrane proximal cysteine residue, followed by the linker GDLVPRGSSRLD (*15*) that contained a sequence cleaved by thrombin (*29*), followed by the ζ chain transmembrane and cytoplasmic domains. Expressed constructs that had linker sequences GD or LVPRGSD (*15*) could not be cleaved by thrombin, although cells transfected with either of these constructs expressed large amounts of cell surface heterodimer and could be activated by antigen-MHC (*10*).

The large amount of surface expression of these constructs presumably reflects enhanced assembly and transport out of the ER. By means of pulse-chase endoglycosidase H (endo H) analysis, we compared the efficiency of ER to Golgi transport in cloned RBL-2H3 transfectants that expressed either both α-ζ and β-ζ chains or only the α-ζ chain. Cells were pulsed with ^{35}S-labeled methionine and then incubated in complete media for 0, 2, or 6 hours. Lysates from these cells were then precipitated with the rabbit antiserum 386 (anti-ζ) (*12*) and incubated either with or without endo H. The resulting samples were analyzed by SDS-PAGE under reducing and nonreducing conditions. In a clone that expressed both α-ζ and β-ζ chains, much of the precipitated material became resistant to endo H digestion (Fig. 2, A and B). Although the clone that expressed only the α-ζ protein formed covalent homodimers, no endo H–resistant material could be detected. Pulse-chase endo H analysis of cells that expressed the neutral transmembrane α and β constructs demonstrated that most chains did not form disulfide-linked dimers (Fig. 2C) and did not exit the ER (*10*). Thus, the ζ chain domain is essential for efficient dimerization of the chimeras, and only heterodimers are efficiently transported out of the ER.

The RBL-2H3 cells that express chimeric constructs that encode ζ cytoplasmic domains are activated by cross-linking of the extracellular domains of these chimera, an event measured by serotonin exocytosis (*5*). We determined whether RBL-2H3 transfectants that express α-ζ/β-ζ heterodimers can be activated by exposure to a specific antigen-MHC complex, which for the 2B4 TCR is a cytochrome c–derived peptide bound to an I-E^k–encoded MHC molecule (*13*). Transfectants were exposed to an E^k-expressing cell line that had been preincubated with a cytochrome c peptide analog or a peptide that bound E^k but was not recognized by 2B4. Serotonin was released specifically upon exposure of the transfectants to cells that presented the appropriate antigen-MHC complex, thus demonstrating that α-ζ/β-ζ heterodimers are capable of recognizing antigen-MHC. These data also show that the αβ heterodimer can recognize antigen-MHC in the absence of the CD3 complex.

To convert the transmembrane-spanning heterodimer into a soluble form, we inserted a linker that contained a thrombin cleavage site on the NH_2-terminal side of the transmembrane domain, such that thrombin treatment could release soluble heterodimer. To demonstrate solubilization, we precipitated α-ζ/β-ζ dimers from surface ^{125}I-labeled cells onto protein A–Sepharose beads. The material was treated with or without thrombin, and then both the supernatant and the bead-bound protein were collected and analyzed by SDS-PAGE under reducing and nonreducing conditions. Digestion with thrombin resulted in the disappearance of almost all intact α-ζ/β-ζ protein from the bead-bound material, and the appearance of material in the supernatant that migrated at these positions was consistent with that expected for a disulfide-linked αβ heterodimer truncated after the membrane proximal cysteine residue. Most of the thrombin-released material ran as a dimer under nonreducing conditions, which demonstrated that the ζ domain induces interchain disulfide linkage directly between the α and β domains as well as through the ζ transmembrane. A parallel digestion of α-ζ/β-ζ protein that lacked thrombin sites yielded only small amounts of proteolytic products, which indicates that thrombin treatment does not result in nonspecific degradation (*10*). This method of solubilization allows for affinity purification followed by release under nondenaturing conditions, in contrast to the purification protocols described for other soluble αβ heterodimers (*3*).

The attachment of the ζ chain transmembrane and cytoplasmic domains to the α and β chain extracellular domains thus provides for the efficient production and cell surface expression of αβ heterodimers and is a means by which to test the functional integrity of the expressed heterodimer. This approach has also been used to obtain a large amount of surface expression of two human αβ heterodimers in addition to 2B4 (*10*) and thus appears generalizable. Inclusion of protease cleavage sites makes the production of a soluble αβ heterodimer feasible, which in turn may allow for a number of structural studies of the heterodimer, including crystallographic analysis and measurements of the affinity between TCR αβ, MHC, and antigen. We anticipate that either soluble heterodimers or RBL-2H3 transfectants could also be used to generate antibodies to TCRs that recognize native epitopes. This approach could be incorporated into strategies for the efficient expression of other protein dimers.

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Prevention of Autoimmune Diabetes in the BB Rat by Intrathymic Islet Transplantation at Birth

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Spontaneous diabetes in the BioBreeding (BB) rat, like human type I diabetes, results from the destruction of pancreatic islets by autoreactive T lymphocytes recognizing β cell–specific antigens. T cell tolerance is in part mediated by interactions of maturing thymocytes with antigens expressed in the thymic microenvironment; islets were therefore implanted into the thymus of neonatal diabetes-prone BB rats to determine whether exposure of T cell precursors to β cell antigens could influence the development of diabetes. This treatment completely prevented diabetes and insulitis in the native pancreas. The effect may be the result of specific modulation of diabetogenic T cells maturing in an islet-bearing thymus.

Rat pancreatic islet allografts transplanted into the thymus of allogeneic hosts survive indefinitely without the need for chronic immunosuppression. Recipients of these grafts are specifically unresponsive to extrathymic islets transplanted from the same strain, possibly as a result of the deletion or functional inactivation of donor-specific alloreactive clones (*1, 2*). This approach might also be used to alter T cell–mediated immunity to tissue-specific self antigens.

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We examined whether implantation of a small amount of islet tissue into the thymus of neonatal BB rats might prevent the development of autoimmune insulitis in the pancreas, a lesion that ordinarily causes 40 to 60% of these animals to become severely diabetic in young adulthood (*3–7*).

Litters of diabetes-prone BB rats [major histocompatibility complex (MHC) haplotype RT1^u] were separated at birth into randomly selected experimental and control groups. Each member of the experimental half-litters received an intrathymic inoculum of 60 to 80 islets (30 to 40 per thymic lobe) isolated from the pancreata of MHC-compatible Wistar Furth (WF,