Restoration of T Cell Development in RAG-2–Deficient Mice by Functional TCR Transgenes

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Introduction of TCRα transgene, TCRβ transgene, or both into RAG-2$^{-/-}$ mice differentially rescues T cell development. RAG-2$^{-/-}$ mice have small numbers of TCR$^-$CD4$^-$CD8$^-$ (double negative, DN) thymocytes that express CD3γδε and ζ proteins intracellularly. Introduction of a TCRβ transgene, but not a TCRα transgene, into the RAG-2$^{-/-}$ background restored normal numbers of thymocytes. These cells were CD4$^+$CD8$^+$ (double positive, DP) and expressed small amounts of surface TCRβ chain dimers in association with CD3γδε but not ζ. RAG-2$^{-/-}$ mice that expressed α and β TCR transgenes developed both DP and single positive thymocytes. Thus, the TCRβ subunit, possibly in association with a novel CD3 complex, participates in the DN to the DP transition.

Genes that encode the variable regions of immunoglobulin (Ig) and T cell receptor (TCR) molecules are assembled during the early stages of T and B cell development by a site-specific recombination process referred to as V(D)J (V, variable; D, diversity; J, joining) recombination (*1*). The complete antigen receptor complex comprises either Ig (on B cells) or TCR (on T cells) chains that bind antigen in association with additional subunits that have been implicated in signal transduction (*2, 3*). For the αβ TCR, this complex consists of a disulfide-linked αβ heterodimer that is noncovalently associated with invariant CD3 chains (γ, δ, ε, and ζ, with or without η). Expression of TCRαβ heterodimers on the cell surface requires the associated CD3 components (*4*).

Immature T lineage cells progress from a CD4$^-$CD8$^-$ double negative (DN) stage to a CD4$^+$CD8$^+$ double positive (DP) stage and, subsequently, to the mature CD4$^+$ or CD8$^+$ single positive (SP) stage (*5*). Expression of complete αβ TCR and its recognition of self major histocompatibility complex (MHC) molecules is involved in the later stages of T cell development, in particular for mediating the transition from the DP to the SP stage through positive or negative selection in a ligand-dependent manner (*6, 7*). Assembly and expression of TCRβ chain gene generally occurs first and may be involved in effecting allelic exclusion at the TCRβ locus while leading to TCRα chain gene assembly or expression (or both) (*8*). Furthermore, introduction of a functionally rearranged TCRβ transgene into the severe combined immunodeficient (*scid*) background partially rescues T cell development from the DN to the DP stage (*9*). Thus, the β chain may be important at early stages of T cell development, independent of its recognition of ligand when it is associated with TCRα chains in the mature receptor.

Mice that lack mature B and T cells have been created by targeted disruption of either the recombination activation gene–1 (RAG-1) or RAG-2 gene (*10, 11*). The only known defect in RAG-deficient animals is the inability to initiate V(D)J rearrangement. To determine the effect of productively rearranged TCR genes on T cell development in RAG-2$^{-/-}$ mice, we introduced TCRα, TCRβ, or both TCR transgenes into the RAG-2$^{-/-}$ background (*12*). The TCRα and TCRβ transgenes encode, when expressed together, a chicken ovalbumin–specific, MHC class II(I-A^d)–restricted TCR (*13*). Thymuses of RAG-2$^{-/-}$ mice contained, on average, 3.3 × 10^6 thymocytes, all of which were DN in phenotype and lacked detectable surface CD3 expression (*11*) (Fig. 1). In TCRα transgenic, RAG-2$^{-/-}$ mice (TCRα$^+$,RAG-2$^{-/-}$), the thymus was essentially indistinguishable from that of the RAG-2$^{-/-}$ mice (Fig. 1). These mice yielded an average of 7 × 10^6 thymocytes. In contrast, introduction of a productive TCRβ transgene into the RAG-2$^{-/-}$ background (TCRβ$^+$,RAG-2$^{-/-}$) restored the thymocyte number (4.5 × 10^8) to that of normal wild type or RAG-2$^{-/+}$ heterozygotes (3.2 × 10^8). More than 95% of these thymocytes were DP (Fig. 1) and weakly, but clearly, expressed TCRβ and

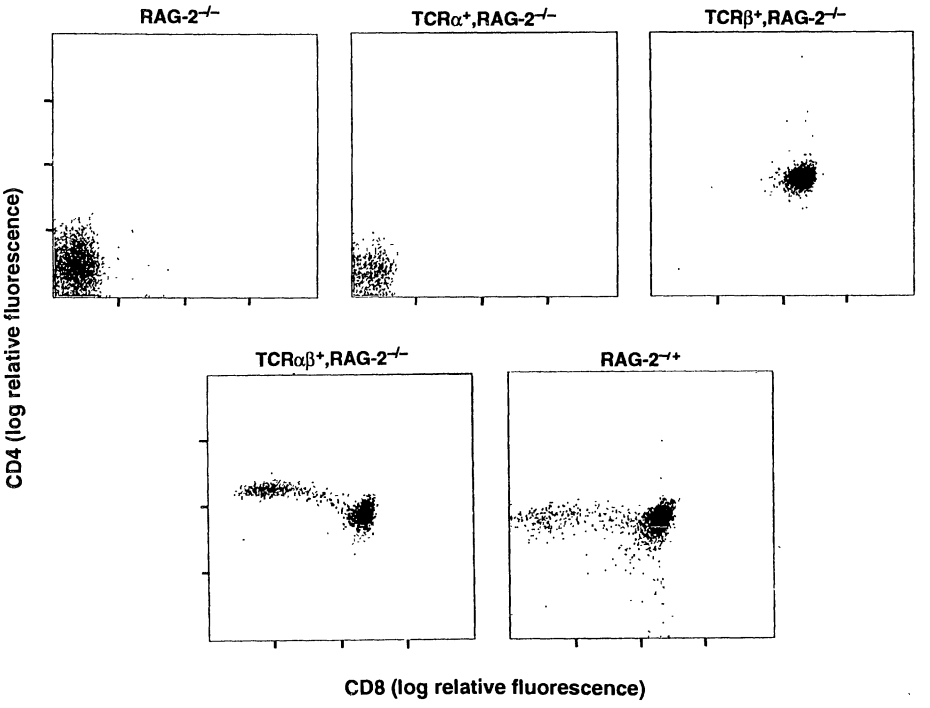

Fig. 1. Rescue of the T cell development in RAG-2$^{-/-}$ mice by functionally rearranged TCRαβ chain genes. Thymocytes obtained from RAG-2$^{-/-}$ and RAG-2$^{-/+}$ mice, and RAG-2$^{-/-}$ mice reconstituted with functionally rearranged TCRα (TCRα$^+$,RAG-2$^{-/-}$), TCRβ (TCRβ$^+$,RAG-2$^{-/-}$), or TCRα plus TCRβ (TCRαβ$^+$,RAG-2$^{-/-}$) genes were immediately examined for CD4 and CD8 expression using fluorescein-coupled MAb 53-6 (anti-CD8α) (*22*) and phycoerythrin (PE)-coupled MAb GK1.5 (anti-CD4) (*23*). Dead cells were excluded by staining with propidium iodide. All analyses were carried out using FACScan (Becton Dickinson, Mountain View, California). The TCRαβ$^+$,RAG$^{-/-}$ shown were *I-A$^{b/d}$*.

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CD3ε chains on their surface (as compared to RAG-$2^{-/+}$ thymocytes, Fig. 2, A to C). These reactivities, as defined by hamster monoclonal antibodies (MAbs) H57 (anti-Cβ) and 500A2 (anti-CD3ε), respectively, are specific because the staining was brighter than that obtained using 3A10, a hamster MAb to mouse Cδ. TCRβ and CD3ε expression were one order of magnitude less than on the DP TCR^{low} subset of normal mice as judged by quantitative immunofluorescence. In addition, there were no detectable SP thymocytes (Fig. 1) or peripheral DP T cells in the TCRβ$^+$,RAG-$2^{-/-}$ mice.

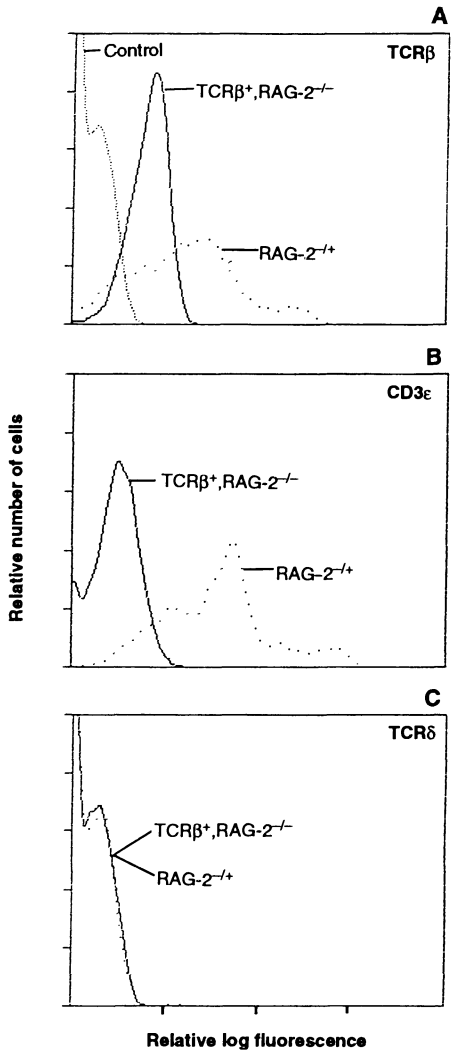

Fig. 2. Surface expression of TCRβ chain and CD3ε on TCRβ transgenic RAG-$2^{-/-}$ mice. Thymocytes from RAG-$2^{-/+}$ mice and TCRβ transgenic RAG-$2^{-/-}$ mice (TCRβ$^+$,RAG-$2^{-/-}$) were examined for (**A**) TCRβ chain, (**B**) CD3ε, and (**C**) TCRδ chain expression on their cell surface. The cells were stained with biotinylated MAbs to TCRCβ (H57) (*24*), CD3ε (500A2) (*25*), and TCRCδ (3A10) (*26*), followed by PE-streptavidin. The control in (A) represents the fluorescence intensity of the TCRβ$^+$,RAG-$2^{-/-}$ thymocytes without biotinylated antibody staining. The 3A10 staining in (C) was indistinguishable from the control staining in (A).

Reconstitution of RAG-$2^{-/-}$ mice with both the TCRα and β transgenes led to essentially complete rescue of all stages of T cell development; both the DP population and a significant population of CD4 SP cells were detectable in the thymus (Fig. 1). Because the transgene-encoded receptor is restricted to I-A^d (*13*), CD4 SP but not CD8 SP mature thymocytes were positively selected in the presence of that MHC element (Fig. 1). We also detected CD4 SP T cells in spleen from the TCRαβ transgenic RAG-$2^{-/-}$ mice; however, no B cells were detected in any of the TCR transgenic, RAG-$2^{-/-}$ mice analyzed. As expected, the RAG-$2^{-/+}$ heterozygous animals had a normal thymic phenotype in terms of DP and SP subpopulations (Fig. 1).

To examine the structure of the surface TCR complex on thymocytes of RAG-$2^{-/-}$ mice, TCRβ transgenic mice and TCRβ$^+$,-RAG-$2^{-/-}$ mice were labeled with [^{35}S]methionine and [^{35}S]cysteine. Cells were lysed with digitonin to preserve any weak associations between TCR and CD3 subunits, and TCR complexes were immunoprecipitated with MAbs 3A10, H57, 2C11 (anti-CD3ε), or a mixture of 1ζ3A1 (anti-CD3ζ) and 1η4F2 (anti-CD3η) (designated as anti-CD3ζ/η). Proteins were analyzed by two-dimensional nonreducing and reducing SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 3). As expected, neither anti-Cδ (Fig. 3, panel a) nor anti-Cβ (Fig. 3A, panel b) precipitated TCR heterodimers from RAG-$2^{-/-}$ thymocytes; however, anti-CD3ε (Fig. 3A, panel c) and anti-CD3ζ/η (Fig. 3A, panel d) MAbs identified intracellular CD3 proteins. Anti-CD3ε immunoprecipitates contained three prominent spots with molecular weights of 21, 25, and 26 kD. The 25-kD protein, which migrated more slowly in reducing conditions than in nonreducing conditions because of intramolecular disulfide bonds, represents CD3ε. The positions of 21-kD and 26-kD proteins are consistent with the known mobilities of CD3γ and CD3δ, respectively. We also observed several off-diagonal spots that represent partial complexes formed between these subunits. For example, the off-diagonal spot of 21 kD in the second dimension and the ~40-kD spot in the first dimension likely represent a previously reported CD3γ dimer (*3*). Anti-CD3ζ/η precipitated a CD3ζ homodimer that migrates off-diagonal and has a molecular size of 16 kD (Fig. 3A, panel d). These results indicate that CD3 subunits are expressed in thymocytes in the absence of rearranged TCRα, β, γ, or δ genes and that CD3γ, δ, and ε form partial complexes unassociated with CD3ζ/η.

From TCRβ transgenic mouse thymocytes, anti-CD3ε immunoprecipitated TCRαβ heterodimers and CD3γδε and CD3ζ homodimers (Fig. 3B, panel c). Anti-Cβ and anti-CD3ζ/η also immunoprecipitated these components (Fig. 3B, panels b and d). Identical results were obtained with thymocytes from nontransgenic normal mice indicating that, as expected, TCRαβ heterodimers are associated with CD3γδε and CD3ζ homodimers in the TCR complex.

Immunoprecipitated products from thymocytes of TCRβ$^{\pm}$,RAG-$2^{-/-}$ mice were quite different from those of TCRβ transgenic and normal mice. From these cells, anti-Cβ immunoprecipitated an off-diagonal spot of ~40 kD (Fig. 3C, panel b). Given its off-diagonal position and the fact that the H57 MAb used recognizes a framework determinant on the constant region of the TCRβ subunit, this spot likely represents a $TCR\beta_2$ homodimer. Alternatively, TCRβ could dimerize with an unknown protein of similar molecular weight. Note that CD3ε (and CD3γ and CD3δ) but not CD3ζ is readily detected in H57 immunoprecipitates (Fig. 3C, panel b). Anti-CD3ε immunoprecipitated the same off-diagonal protein of 40 kD as anti-Cβ, as well as CD3γδε (Fig. 3C, panel c), indicating that the putative $TCR\beta_2$ dimers associate with the CD3γδε. In contrast, CD3ζ is not precipitated by anti-CD3ε. The absence of CD3ζ in the complex is not due to the lack of expression of the CD3ζ because the specific anti-ζ/η MAbs immunoprecipitated the $CD3\zeta_2$ dimer (Fig. 3, panel d). These results indicate that CD3ζ is not detectably associated with the TCRβ-CD3γδε complex.

Expression of transgenic TCRβ chains, but not TCRα chains, in RAG-$2^{-/-}$ mice permits the DN thymocytes to transit to the DP stage and restores normal thymic cellularity. This finding concurs with the observation that introduction of a TCRβ transgene into SCID mice leads to the generation of some DP cells (9). However, unlike TCRβ$^+$,RAG-2–deficient mice, the number of thymocytes in TCRβ transgenic SCID mice remained low (about 2×10^6) and a substantial proportion of these thymocytes remained DN (6). The *scid* defect allows initiation of V(D)J recombination but impairs coding join formation and may, thereby, predispose developing lymphocytes to a high frequency of lethal double-strand breaks (*14*). In addition, the *scid* defect also affects double-strand DNA break repair in both lymphoid and nonlymphoid cells (*15*). Thus, the apparently inefficient maturation of TCR transgenic SCID thymocytes may result from stimulation of endogenous V(D)J recombination events leading to cell death or by unknown pleiotropic effects of the *scid* mutation. In agreement with the conclusion that the inability to initiate V(D)J rearrangement in devel-

oping lymphocytes is the only defect in RAG-2–deficient mice, both early and later stages of T cell development can be totally rescued by the introduction of rearranged TCR transgenes into this background.

CD3 subunits are present in the thymocytes of RAG-$2^{-/-}$ mice. Thus, it is likely that CD3 subunits are expressed intracellularly in early thymocyte development before V(D)J recombination and are transported to the cell surface after assembly and expression of functional TCR chains. We detect a TCRβ-CD3γδε complex on the surface of TCRβ$^+$,RAG-$2^{-/-}$ thymocytes. Because we also detect CD3ζ chains in these cells, its absence from their surface TCRβ complex cannot be attributed to the relative instability of this CD3 component (*16*). Therefore, ability to express the surface TCRβ complex may be related to lack of association of CD3ζ. $TCR\beta_2$ dimers without CD3ζ are present on immature SCID thymocyte cell lines reconstituted with a rearranged TCRβ chain gene (*17*), but were not detected on mature T cells or T cell lines that do not have a TCRα chain. In addition, expression of surface TCRβ chains was not detected in DP thymocytes that accumulate in mice homozygous for a mutation that blocks assembly of functional TCRα chains (*18*). It is conceivable that endogenous TCRβ chains may have been expressed on the surface of these cells but at levels lower than those of our TCRβ transgenes and which were not readily detectable. However, these molecules need to be detected on the surface of normal thymocytes.

We do not yet know why the TCRβ complex was not expressed on the surface of thymocytes that are more mature. One possibility is that additional cellular compo-

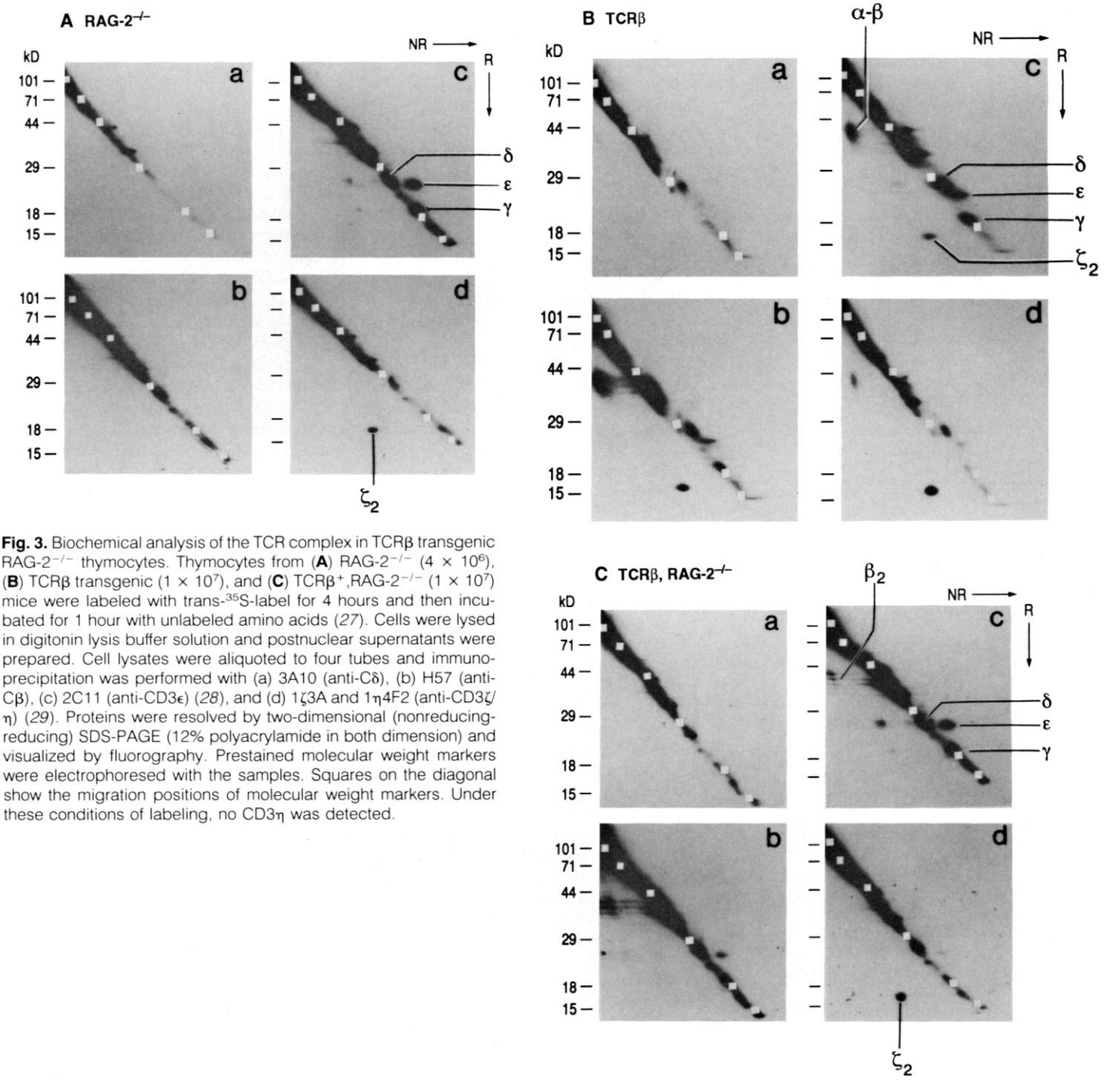

Fig. 3. Biochemical analysis of the TCR complex in TCRβ transgenic RAG-$2^{-/-}$ thymocytes. Thymocytes from (**A**) RAG-$2^{-/-}$ (4×10^6), (**B**) TCRβ transgenic (1×10^7), and (**C**) TCRβ$^+$,RAG-$2^{-/-}$ (1×10^7) mice were labeled with trans-^{35}S-label for 4 hours and then incubated for 1 hour with unlabeled amino acids (*27*). Cells were lysed in digitonin lysis buffer solution and postnuclear supernatants were prepared. Cell lysates were aliquoted to four tubes and immunoprecipitation was performed with (a) 3A10 (anti-Cδ), (b) H57 (anti-Cβ), (c) 2C11 (anti-CD3ε) (*28*), and (d) 1ζ3A and 1η4F2 (anti-CD3ζ/η) (*29*). Proteins were resolved by two-dimensional (nonreducing-reducing) SDS-PAGE (12% polyacrylamide in both dimension) and visualized by fluorography. Prestained molecular weight markers were electrophoresed with the samples. Squares on the diagonal show the migration positions of molecular weight markers. Under these conditions of labeling, no CD3η was detected.

nents present only in immature thymocytes play a role in transportation of the TCRβ-CD3γδε complex to the cell surface. An alternative is that such partial complexes were degraded more rapidly in mature T cells. Our results also suggest that the TCRα subunit is important for the association of CD3ζ subunits with a TCR. In T cell hybridomas lacking CD3ζ/η, a small amount of TCRαβ-CD3γδε surface expression has been observed (*19*). Transfection of CD3ζ, η, or both up-regulated surface expression of the TCR in these mutants, demonstrating the importance of CD3ζ/η subunits in transportation of the TCR complex (*20*).

The potential function of the TCRβ chain surface complex in early T cell development remains to be determined. In precursor B cells, it has been proposed that a surface complex that includes the Ig heavy chain in association with surrogate light chains participates in signaling the allelic exclusion of Ig heavy chain and onset of the assembly of Ig light chain V genes (*21*). It is possible that the TCRβ-CD3γδε complex identified by the immunoprecipitation studies herein could function in signaling such regulatory events. Finally, the ability of the TCRβ transgene to promote the phenotypic conversion of DN to DP cells and expand the pool of thymocytes in RAG-$2^{-/-}$ mice likely reflects an important normal physiological function of this TCR subunit that should now be readily amenable to further analysis.

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β-Adrenergic Receptor Kinase–2 and β-Arrestin–2 as Mediators of Odorant-Induced Desensitization

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β-Adrenergic receptor kinase (βARK) and β-arrestin function in the homologous or agonist-activated desensitization of G protein–coupled receptors. The isoforms βARK-2 and β-arrestin–2 are highly enriched in and localized to the dendritic knobs and cilia of the olfactory receptor neurons where the initial events of olfactory signal transduction occur. Odorants induce a rapid and transient elevation of adenosine 3′,5′-monophosphate (cAMP), which activates a nonspecific cation channel and produces membrane depolarization. Preincubation of rat olfactory cilia with antibodies raised against βARK-2 and β-arrestin–2 increased the odorant-induced elevation of cAMP and attenuated desensitization. These results suggest that βARK-2 and β-arrestin–2 mediate agonist-dependent desensitization in olfaction.

The primary events of olfactory signal transduction occur in the cilia of olfactory receptor neurons and lead to the generation of the intracellular second messengers cAMP and inositol 1,4,5-trisphosphate (IP_3) (*1–3*). Isoforms of several components of these transduction pathways are highly enriched in olfactory neuronal cilia and include a type III adenylyl cyclase (*4*), a cyclic nucleotide–gated nonspecific cation channel (*5*), and an α subunit of a stimulatory G protein, G_{olf} (*6*). The membrane receptors presumed to transduce the odorant stimulus are members of a large family of G protein–linked receptors (*7*) and are localized in the olfactory neuron too (*8*).

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As with other responses mediated by G protein–coupled receptors, odorant-stimulated signals attenuate rapidly even in the continual presence of stimulus, a phenomenon termed desensitization. Although the initial events of olfactory signal transduction have been extensively explored, the mechanisms of desensitization to odorants are not known.

Desensitization of signal transduction may occur through a variety of processes, including receptor internalization or receptor uncoupling mediated by receptor phosphorylation (*9*). Homologous or agonist-induced desensitization of the β_2-adrenergic receptor occurs through agonist-activated receptor phosphorylation that is catalyzed by a specific receptor kinase called β-adrenergic receptor kinase (βARK) (*10, 11*). Further quenching of signal transduction requires the binding of the protein β-arrestin to phosphorylated receptors (*12*). Three receptor kinases—rhodopsin kinase (*13*), βARK-1 (*12*), and βARK-2 (*14*)—have been identified and three arrestins are known—arrestin (*15*), β-arrestin–1 (*12*), and β-arrestin–2 (*16*). Localiza-