Src-family PTKs (8) and Syk PTK (9). Thus, IL-2R components recruit one or more PTKs, all of which may be required for maximum signaling. Finally, our experiments with reconstituted IL-2R provide functional evidence for the involvement of Jaks in the growth signal transmission by cytokines.

REFERENCES AND NOTES

1. K. A. Smith, *Science* **240**, 1169 (1988); T. A. Waldmann, *Annu. Rev. Biochem.* **58**, 875 (1989); Y. Minami, T. Kono, T. Miyazaki, T. Taniguchi, *Ann. Rev. Immunol.* **11**, 245 (1993); T. Taniguchi and Y. Minami, *Cell* **73**, 5 (1993); W. J. Leonard, M. Noguchi, S. M. Russell, O. W. McBride, *Immunol. Rev.* **138**, 61 (1994).
2. D. Cosman *et al.*, *Trends Biochem. Sci.* **15**, 265 (1990); J. F. Bazan, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 6934 (1990).
3. M. Hatakeyama, H. Mori, T. Doi, T. Taniguchi, *Cell* **59**, 837 (1989).
4. Y. Nakamura *et al.*, *Nature* **369**, 330 (1994); B. H. Nelson, J. D. Lord, P. D. Greenberg, *ibid.*, p. 333.
5. A. Kawahara, Y. Minami, T. Taniguchi, *Mol. Cell. Biol.* **14**, 5433 (1994).
6. M. Noguchi *et al.*, *Cell* **73**, 147 (1993).
7. W. L. Farrar and D. K. Ferris, *J. Biol. Chem.* **264**, 12562 (1989); M. R. Fung *et al.*, *J. Immunol.* **147**, 1253 (1991).
8. M. Hatakeyama *et al.*, *Science* **252**, 1523 (1991); N. Kobayashi *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 4201 (1993); Y. Minami *et al.*, *EMBO J.* **12**, 759 (1993).
9. Y. Minami and T. Taniguchi, in preparation.
10. B. A. Witthuhn *et al.*, *Nature* **370**, 153 (1994).
11. J. A. Johnston *et al.*, *ibid.*, p. 151.
12. Anti-Jak1, anti-Jak2, anti-Jak3, or anti-CD4 immunoprecipitates were resuspended in 30 μl of the kinase buffer [20 mM Hepes (pH 7.2), 10 mM $MnCl_2$, 30 μM Na_3VO_4, 0.1% (v/v) Brij 96] containing 10 μCi of [γ-^{32}P]ATP (adenosine triphosphate) (5000 Ci/mmol; Amersham) and incubated for 15 min at 25°C. The reaction was terminated by the addition of Laemmli buffer. Subsequently, samples were subjected to SDS–polyacrylamide gel electrophoresis (PAGE) (7.5%) under reducing conditions and were transferred to polyvinylidene difluoride membrane filters (Immobilon, Millipore). Subsequently, membrane filters were treated with 1 N NaOH for 1 hour at 65°C, fixed, and subjected to autoradiography. Similar to the results obtained by immunoblot analysis, in vitro kinase assays revealed that the cytoplasmic S region of IL-2Rβ and the COOH-terminal 48 amino acids within IL-2Rγ are required for association with Jak1 and Jak3, respectively.
13. Metabolic labeling experiments of COS cells with [^{35}S]Met and [^{35}S]Cys were done as described (*24*). Expression of the CD4–IL-2R chimeras in COS cells was found to be comparable when the radioactivity of the band corresponding to respective chimeras was normalized by methionine-cysteine content (quantitated with a Fujix imaging analyzer, BAS 2000). Expression of the respective Jaks in transfected COS cells was also comparable in the results of immunoblot analysis of these cell lysates with antisera against the respective Jaks.
14. The chimeric receptors CD4β, CD4γ, and CD4γM1 were stably transfected into an IL-3–dependent cell line, BAF-B03, and clonal lines expressing comparable levels of chimeric proteins were used. When cell lysates from these lines were immunoprecipitated with anti-CD4 and subjected to immunoblotting with anti-Jak1, anti-Jak2, or anti-Jak3, selective coimmunoprecipitation of Jak1 with CD4β, and Jak3 with CD4γ, but not CD4γM1, was observed. In contrast, Jak2 was coimmunoprecipitated with neither of these chimeric receptors.
15. A. Kawahara, H. Fujii, Y. Nakagawa, T. Miyazaki, unpublished observations.
16. M. Kondo *et al.*, *Science* **262**, 1874 (1993); M. Noguchi *et al.*, *ibid.*, p. 1877; S. M. Russell *et al.*, *ibid.*, p. 1880; M. Kondo *et al.*, *ibid.* **263**, 1453 (1994).
17. S. M. Zurawski, F. Vega Jr., B. Huyghe, G. Zurawski, *EMBO J.* **12**, 2663 (1993); J. G. Giri *et al.*, *ibid.* **13**, 2822 (1994).
18. M. Murakami *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 11349 (1991); R. Fukunaga *et al.*, *EMBO J.* **10**, 2855 (1991).
19. B. A. Witthuhn *et al.*, *Cell* **74**, 227 (1993).
20. Y. Minami *et al.*, *J. Immunol.* **152**, 5680 (1994).
21. Jak3 cDNA (*10*) was inserted into pEF expression vector (*5*). This expression vector and neomycin-resistance gene (pST neoB) were cotransfected into 3T3αβγ cells by the calcium phosphate method (*20*). Selection was initiated 48 hours after the DNA transfection, using neomycin (700 μg/ml) in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum (FCS). 3T3αβγ-derived transformants, J3-1, J3-12, and J3-13 clones, which express Jak3, were obtained.
22. N. Tanaka *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 7271 (1994).
23. B. A. Witthuhn and J. N. Ihle, unpublished data.
24. T. Miyazaki *et al.*, *EMBO J.* **10**, 3191 (1991).
25. K. Yamamoto *et al.*, *Mol. Cell. Biol.* **14**, 4342 (1994).
26. COS cells and BAF-B03–derived cells were solubilized with lysis buffer [10 mM tris-HCl (pH 7.5), 150 mM NaCl, 2 mM Na_3VO_4, 1 mM *p*-amidinophenyl methanesulfonyl fluoride hydrochloride (pAPMSF), aprotinin (10 μg/ml), 1% (v/v) Brij 96] for 30 min at 4°C. The lysates were centrifuged to remove insoluble materials and the resultant supernatants were then precleared by incubation for 1 hour at 4°C with protein A–Sepharose (Pharmacia). The precleared supernatants were then immunoprecipitated with the respective antibodies (OKT4, anti-Jak1, anti-Jak2, and anti-Jak3) and protein A–Sepharose for 2 hours at 4°C.
27. O. Silvennoinen *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 8429 (1993).

28. We thank T. Saito for the anti-human CD4 (OKT4), H. Nakauchi for pBS-hCD4, M. Lamphier for critical reading of the manuscripts, and H. Shibuya, T. Matsuda, and T. Shirasawa for helpful discussions. Supported in part by a Grant-in-Aid for Special Project Research, Cancer Bioscience, from the Ministry of Education, Science, and Culture of Japan and in part by National Cancer Institute Center Support (CORE) grant P30 CA21765, and by grant RO1 DK42932 from the National Institute of Diabetes and Digestive and Kidney Diseases and the American Lebanese-Syrian Associated Charities.

8 August 1994; accepted 19 September 1994

Role of TCR ζ Chain in T Cell Development and Selection

Elizabeth W. Shores,* Kun Huang, Tom Tran, Eric Lee, Alexander Grinberg, Paul E. Love

Signals mediated by the T cell receptor (TCR) are required for thymocyte maturation and selection. To examine the role of TCR ζchain signals in development, TCR expression was restored in ζ-deficient mice with transgenic ζ chains that partially or completely lacked sequences required for signal transduction. The ζ chain played a role in thymic development by promoting TCR surface expression, but ζ-mediated signals were not essential because TCRs that contained signaling-deficient ζ chains promoted T cell maturation and transduced signals associated with thymic selection.

Differentiation of precursor thymocytes into mature, functional T cells is a multistep process controlled by signals delivered through the TCR (*1*). The TCR is composed of at least six different subunits that function either in antigen recognition or in signal transduction (*2*). The clonotypic TCRαβ (or TCRγδ) chains are responsible for ligand specificity, lack inherent signaling activity, and associate noncovalently with multiple signal-transducing subunits: the CD3γ, CD3δ, and CD3ε components and a dimer composed of one or more members of the ζ family of proteins [ζ, η, or the γ chain of the type I immunoglobulin (Ig) E Fc receptor, FcεR1γ] (*3*). The CD3 and ζ family proteins contain partially conserved sequences in their cytoplasmic domains, called tyrosine-based activation motifs (TAMs), that couple the TCR to intracellular signal transduction pathways (*4*). These motifs are not identical, and it has been suggested that they may recruit distinct signal transduction molecules (*4*, *5*). The CD3 chains each contain a single TAM, whereas ζ contains three TAMs and is thought to represent the predominant TCR signaling structure.

Thymocyte development is severely affected in ζ-deficient ($\zeta^{-/-}$) mice: TCR surface expression is barely detectable, $CD4^+CD8^-$ and $CD4^-CD8^+$ [single-positive (SP)] thymocytes are markedly decreased (<1% of control), and few T cells are found in the periphery (*6*, *7*). However, because ζ is required for efficient surface expression of the other TCR subunits including the CD3 signaling molecules, the role of ζ-mediated signals in development could not be directly assessed in $\zeta^{-/-}$ mice. To determine the importance of

E. W. Shores and T. Tran, Division of Hematologic Products, Center for Biologics, Food and Drug Administration, Bethesda, MD 20892, USA.
K. Huang, E. Lee, A. Grinberg, P. E. Love, Laboratory of Mammalian Genes and Development, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892, USA.

*To whom correspondence should be addressed.

ζ-specific signals, we examined thymocyte maturation in $\zeta^{-/-}$ mice reconstituted with transgenes encoding natural or genetically engineered variants of ζ that restore TCR surface expression but that differ in their ability to transduce signals.

The construction of transgenes that encoded full-length ζ; η, a naturally expressed splice variant of ζ; and the truncation variant ζ–D108-164 (amino acid residues 108 through 164 deleted) has been described (Fig. 1) (8, 9). For this investigation, we also generated transgenes that encoded deletion variants ζ–D66-114 and ζ–D67-150 (Fig. 1). Because at least one intact TAM is required for signal transduction (*10*, *11*), the ζ–D67-150, without a TAM, directed the synthesis of a signaling-deficient ζ chain. All of the transgenes were under the control of the human CD2 promoter and enhancer, which confers high-level copy number–dependent expression specifically in the T cell lineage (*12*). Multiple founder lines were obtained for each construct, and the transgenes were subsequently mated into the $\zeta^{-/-}$ background (in which expression of both endogenous ζ and η is abolished) (6) to obtain the transgenic (Tg) lines ($\zeta^{-/-;Tg}$).

Surface TCR expression on thymocytes from the various $\zeta^{-/-;Tg}$ lines was examined by two-dimensional SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2). Thymocytes from unreconstituted $\zeta^{-/-}$ mice expressed small amounts of partial, ζ-deficient TCR complexes on their surface (*13*). In contrast, immunoprecipitates from surface-radioiodinated $\zeta^{-/-;Tg}$ thymocytes demonstrated that the transgenes encoded ζ chain or ζ chain variant proteins of the predicted sizes that dimerized, assembled with other TCR subunits, and promoted the surface expression of intact TCR complexes. Immunofluorescence and flow cytometry (FCM) confirmed the expression of surface TCRs on thymocytes and peripheral T cells from transgene-reconstituted $\zeta^{-/-}$ mice (Fig. 3). Analysis of multiple founder lines for each transgene revealed that the amount of surface TCR increased in proportion to the copy number and expression of the transgenes (*13*). For this investigation, we analyzed Tg founder lines with comparable surface TCR expression (Fig. 3).

To assess T cell development in $\zeta^{-/-;Tg}$ mice, we examined CD4 and CD8 expression on thymocytes and lymph node T cells. In $\zeta^{-/-}$ mice, the numbers of $CD4^+CD8^+$ [double-positive (DP)] thymocytes were reduced, and the numbers of the SP thymocytes were especially reduced (Fig. 3 and Table 1). Expression of Tg full-length ζ chain restored T cell development in $\zeta^{-/-}$ mice (Fig. 3 and Table 1) (6). Each of the ζ chain variants, whether they had zero, one, or two TAMs, also promoted T cell maturation (Fig. 3 and Table 1). Total thymocyte numbers, which reflected predominantly DP thymocytes, were increased 3- to 10-fold in $\zeta^{-/-;Tg}$ relative to $\zeta^{-/-}$ mice (Table 1). Importantly, SP thymocytes were increased 25- to 200-fold (Fig. 3 and Table 1), and peripheral T cells were increased more than 5-fold (*13*) in $\zeta^{-/-;Tg}$ mice relative to $\zeta^{-/-}$ mice. Regardless of the particular

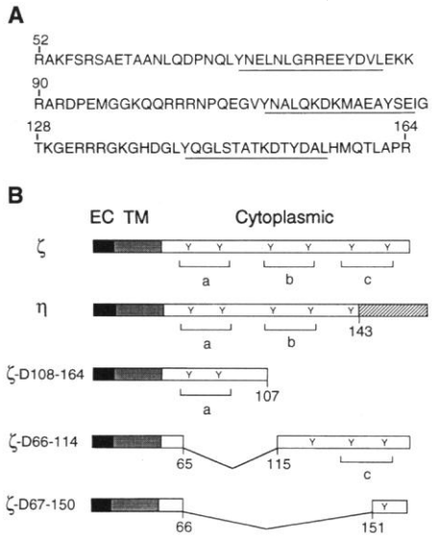

Fig. 1. (**A**) Amino acid sequence of the murine ζ chain cytoplasmic domain. The conserved TAMs (YXXL/IX_{7-8}YXXL/I; a slash indicates L or I at the indicated position) are underlined. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; X, any amino acid; and Y, Tyr. (**B**) Schematic representation of coding sequences in the various transgene constructs. The extracellular (EC), transmembrane (TM), and cytoplasmic domains are depicted in black, shaded, or open boxes, respectively. The hatched box at the COOH-terminus of the η chain represents the unique region generated by alternative splicing. The location of TAMs (a through c) in the cytoplasmic domains of Tg proteins and their tyrosine residues are indicated.

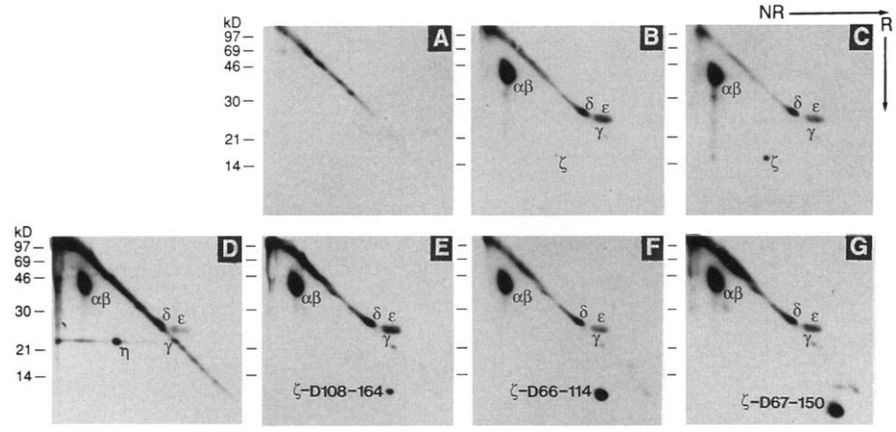

Fig. 2. Subunit composition of TCR complexes expressed on thymocytes from $\zeta^{+/+}$, $\zeta^{-/-}$, and $\zeta^{-/-;Tg}$ mice. (**A**) $\zeta^{-/-}$, (**B**) $\zeta^{+/+}$, (**C**) $\zeta^{-/-;\zeta Tg}$, (**D**) $\zeta^{-/-;\eta Tg}$, (**E**) $\zeta^{-/-;\zeta\text{-D108-164Tg}}$, (**F**) $\zeta^{-/-;\zeta\text{-D66-114Tg}}$, and (**G**) $\zeta^{-/-;\zeta\text{-D67-150Tg}}$. We surface-radioiodinated total thymocytes (*17*), solubilized them in 1% digitonin lysis buffer to maintain intact TCR complexes, and then immunoprecipitated the proteins with antibody 551 to ζ ($\zeta^{+/+}$, $\zeta^{-/-;\zeta Tg}$, $\zeta^{-/-;\zeta\text{-D66-114Tg}}$, and $\zeta^{-/-;\zeta\text{-D67-150Tg}}$), antibody 528 to ζ ($\zeta^{-/-;\eta Tg}$ and $\zeta^{-/-;\zeta\text{-D108-164Tg}}$), or both antibodies ($\zeta^{-/-}$). Immunoprecipitates were resolved by two-dimensional NR-R SDS-PAGE and visualized by autoradiography. Migration positions of the various TCR subunits are indicated. Molecular size standards are shown at left.

Table 1. Numbers of thymocytes in $\zeta^{+/+}$, $\zeta^{-/-}$ and $\zeta^{-/-;Tg}$ mice. Data are given in 10^6 cells as means ± SEM for at least six mice per genotype. Thymocytes were obtained from age-matched 4- to 8-week-old mice. We calculated subpopulations by multiplying the total thymocyte number by the percent of cells in a given quadrant as depicted in Fig. 3.

Genotype	Total	$CD4^-CD8^-$	$CD4^+CD8^+$	$CD4^+CD8^-$	$CD4^-CD8^+$
$\zeta^{+/+}$	219 ± 4	6.8 ± 2.0	186 ± 44.7	17.6 ± 4.6	4.8 ± 3.1
$\zeta^{-/-}$	24 ± 20	4.7 ± 3.3	20 ± 16.5	0.11 ± 0.09	0.05 ± 0.05
$\zeta^{-/-;\zeta Tg}$	259 ± 103	6.3 ± 3.6	217 ± 87.1	24.6 ± 10.5	4.9 ± 2.3
$\zeta^{-/-;\eta Tg}$	72 ± 34	4.7 ± 1.6	54 ± 27.7	6.9 ± 3.8	2.0 ± 0.8
$\zeta^{-/-;\zeta\text{-D108-164Tg}}$	192 ± 59	5.6 ± 2.1	168 ± 59.0	11.0 ± 4.2	2.7 ± 0.5
$\zeta^{-/-;\zeta\text{-D66-114Tg}}$	118 ± 51	3.4 ± 1.0	99 ± 45.0	9.0 ± 4.8	2.1 ± 0.8
$\zeta^{-/-;\zeta\text{-D67-150Tg}}$	96 ± 38	3.9 ± 0.9	84 ± 38.7	4.6 ± 2.3	1.4 ± 0.5

construct, rescue of thymocyte development was transgene dose-dependent and directly paralleled the amount of TCR surface expression (*13*). Consistent with this observation, $\zeta^{-/-}$ mice that express endogenous η but only small amounts of TCR do not efficiently generate SP thymocytes (*14*). Thus, although ζ plays a role by promoting surface expression of the TCR, signals delivered by ζ are not absolutely required for T cell development. These results also suggest that signals transduced by the CD3 subunits alone are sufficient for T cell maturation.

Although the ζ chain signaling motifs were not absolutely required, their presence appeared to augment T cell development as demonstrated by the comparison between mice expressing full-length ζ (three TAMs) and those expressing ζ–D67-150 (zero TAMs) (Fig. 3 and Table 1). Although thymocytes from $\zeta^{-/-;\zeta\text{-D67-150Tg}}$ mice expressed the largest amounts of TCRs, these mice generated the fewest SP cells, whereas mice expressing the full-length ζ chain generated the largest number of SP cells (Fig. 3 and Table 1). Together, these data suggest that ζ chain TAMs can participate in thymocyte development, presumably by contributing to the overall signaling potential of the TCR complex, but are not required for the generation of SP T cells.

We next assessed the ability of the various reconstituted TCRs to transduce signals associated with positive selection. Positive selection of DP thymocytes is associated with the coupling of TCR-mediated signals to protein kinase C (PKC)–dependent CD69 expression (*15*). Cross-linking of TCRs with antibody to TCRβ, but not with a control antibody to CD28, resulted in an increase in CD69 on DP thymocytes from all of the transgene-reconstituted lines (Fig. 4), indicating that ζ chain signaling is not required to couple thymocyte TCRs to the PKC activation pathway.

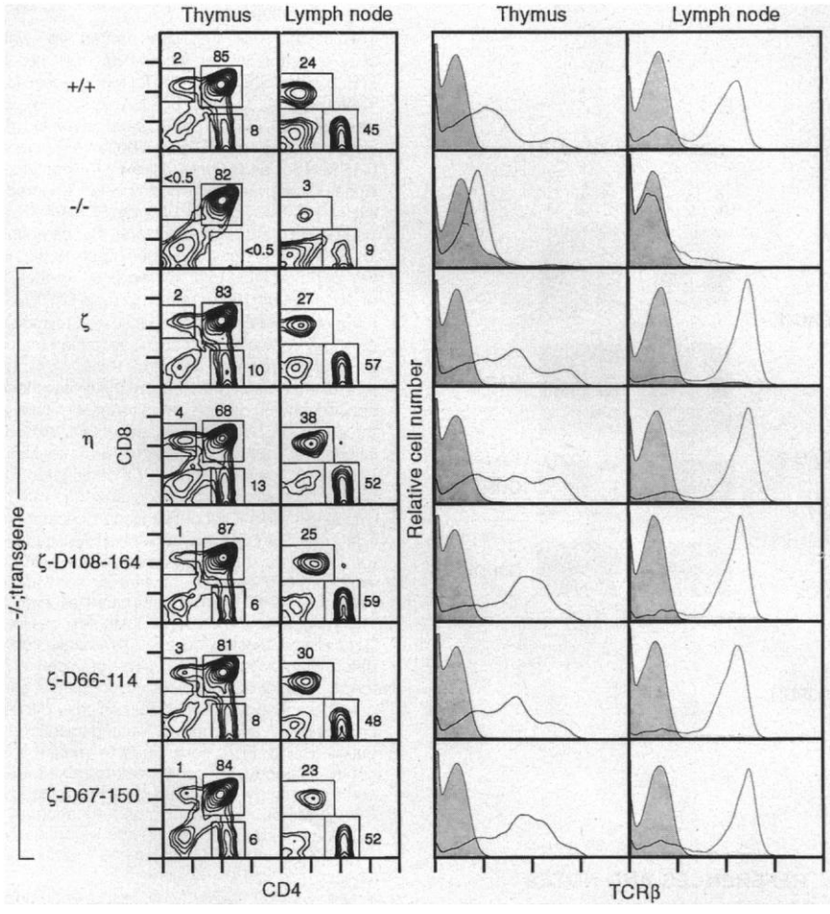

Fig. 3. Analysis of thymocytes and lymph node T cells in $\zeta^{-/-;Tg}$ mice. Shown are immunofluorescence and flow cytometric analyses of cells from young adult (4- to 6-week-old) $\zeta^{-/-}$ mice into which had been bred transgenes encoding full-length or variant ζ chains. Cells were stained with directly labeled mAbs to CD4, CD8, or TCRβ (*18*). For two-color plots, the numbers in quadrants represent the percent of cells contained in that quadrant. For one-color histograms, the shaded areas represent staining with control antibody (PE conjugated to mouse IgG2A). Lymph nodes were isolated from the same mice whose thymocyte profiles are shown.

TCR signaling in thymocytes also results in down-regulation of transcripts encoded by the recombination activating genes RAG-1 and RAG-2 (*16*). To assess the ability of TCR complexes to regulate RAG expression, we cross-linked the TCR DP thymocytes with antibody to TCRβ for 6 hours, after which the expression of RAG-1 and RAG-2 transcripts was examined by Northern (RNA) blot analysis (Fig. 5A). RAG-1 and RAG-2 mRNAs decreased in DP thymocytes from both $\zeta^{+/+}$ and $\zeta^{-/-;Tg}$ mice in response to TCR cross-linking but not when cells were cultured on plates that had been treated with phosphate-buffered saline (PBS) alone (Fig. 5A) or control antibody to CD28 (*13*). The reduction in RAG transcripts was not simply due to induction of cell death because the identical signals resulted in an increased expression of the RNA encoding CD5 (Fig. 5A). Thus, TCR complexes that include ζ chain variants that are either impaired or deficient in signaling potential are competent to transduce physiologically relevant signals and are sufficient to promote the development of mature SP T cells.

The discovery that T cell development can proceed in the absence of ζ chain signaling prompted us to examine the signaling potential of TCR complexes on DP thymocytes from $\zeta^{-/-}$ mice. Thymocytes from $\zeta^{-/-}$ mice express barely detectable surface TCR (Fig. 3) (*6*, *7*). Surface labeling and immunoprecipitation revealed that these complexes are composed of $\alpha\beta$ heterodimers and CD3γ, CD3δ, and CD3ϵ chains that do not appear to be associated with other potential signaling molecules such as FcϵR1γ (*13*). However, TCR complexes on DP thymocytes from $\zeta^{-/-}$ mice transduced signals that resulted in up-regulation of CD69 surface expression (Fig. 4), up-regulation of CD5 mRNA (Fig. 5B), and down-regulation of RAG mRNAs (Fig. 5B).

We have shown that TCR complexes that contain signaling-deficient ζ chains can promote the generation of mature SP T cells. Our observation that these complexes transduce signals is consistent with other studies in T cell lines (*11*), and we further demonstrated that these signals are relevant to thymocyte selection. Our data suggest that the critical function of ζ in development is its ability to promote TCR surface expression. Although ζ-mediated signals are not absolutely required, they can contribute to development because full-length ζ chains were better able to reconstitute T cell development than those lacking one or more TAMs. These findings support the suggestion that the TAM repeats in the ζ chain function in signal amplification (*10*, *11*). The ability of ζ to amplify TCR signals may be re-

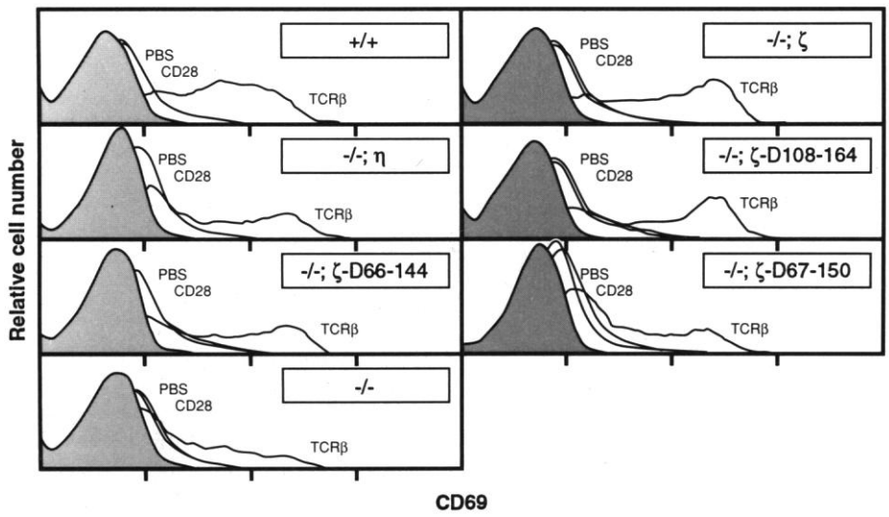

Fig. 4. Induction of surface CD69 expression by $CD4^+CD8^+$ (DP) thymocytes from $\zeta^{-/-;Tg}$ mice after TCR cross-linking. Total thymocytes were incubated at 37°C in plates that had been coated with either PBS, antibody to CD28 (37.51), or antibody to TCRβ (H57-597), as indicated. After 20 hours, the cells were harvested and analyzed by immunofluorescence and three-color FCM. Cells were stained with antibody to CD69 or control mAb (Leu4), followed by staining with antibody to CD4 and antibody to CD8 (*18*). CD69 expression by DP thymocytes was analyzed by software gating. Shaded areas represent control staining on cells incubated with antibody to TCRβ, although we found control staining to be comparable regardless of the cross-linking antibody (*13*).

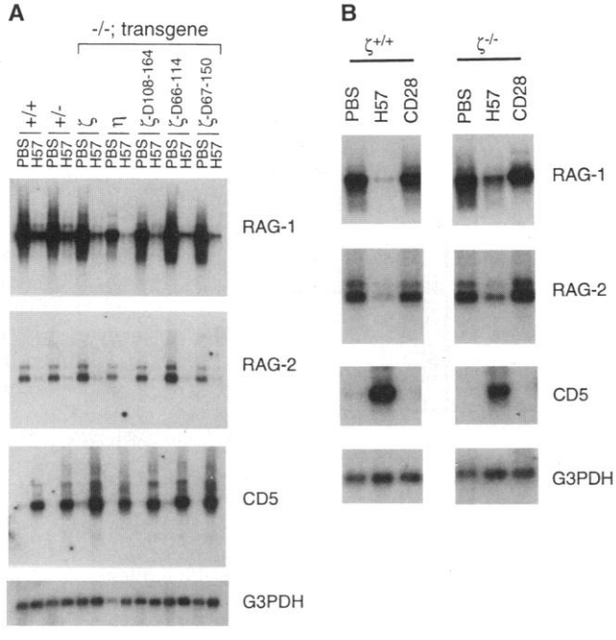

Fig. 5. Regulation of RAG-1, RAG-2, and CD5 expression by TCR cross-linking. $CD4^+CD8^+$ (DP) thymocytes were isolated (*19*) and then incubated at 37°C on plates that had been coated with either PBS, antibody to CD28 (37.51), or antibody to TCRβ (H57-597). After 6 hours, the cells were harvested, and total RNA was extracted and analyzed by Northern blot (*9*) with the indicated complementary DNA probes. To assess loading consistency, we hybridized identical samples with a probe derived from the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) coding region. (**A**) Comparison of RAG-1, RAG-2, and CD5 mRNA in DP thymocytes from $\zeta^{+/+}$ mice and $\zeta^{-/-;Tg}$ mice. (**B**) Comparison of RAG-1, RAG-2, and CD5 mRNA in DP thymocytes from $\zeta^{+/+}$ and $\zeta^{-/-}$ mice.

quired for the selection of thymocytes having TCRs of low avidity. Thus, signals transduced by ζ may influence the specificity of the developing T cell repertoire selected in the thymus. Nevertheless, signals transduced by the CD3 components are themselves sufficient to mediate thymocyte maturation and selection.

REFERENCES AND NOTES

1. S. H. Teh *et al.*, *Nature* **335**, 229 (1988); B. J. Fowlkes and D. Pardoll, *Adv. Immunol.* **44**, 207 (1989); W. C. Sha *et al.*, *Nature* **336**, 73 (1988); H. von Boehmer, *Cell* **76**, 219 (1994).
2. H. Clevers, B. Alarcon, T. Wileman, C. Terhorst, *Annu. Rev. Immunol.* **6**, 629 (1988); R. D. Klausner, J. Lippencott-Schwartz, J. S. Bonifacino, *Annu. Rev. Cell. Biol.* **6**, 403 (1990).
3. D. G. Orloff, C. Ra, S. J. Frank, R. D. Klausner, J. P. Kinet, *Nature* **347**, 189 (1990).
4. M. Reth, *ibid.* **338**, 383 (1989); L. E. Samelson and R. D. Klausner, *J. Biol. Chem.* **267**, 24913 (1992); A. Weiss, *Cell* **76**, 263 (1994).
5. M. Exley, L. Varticovski, M. Peter, J. Sancho, C. Terhorst, *J. Biol. Chem.* **269**, 15140 (1994).
6. P. E. Love *et al.*, *Science* **261**, 918 (1993).
7. M. Malissen *et al.*, *EMBO J.* **12**, 4347 (1993); C.-P. Liu *et al.*, *ibid.*, p. 4863.
8. S. J. Frank *et al.*, *Science* **249**, 174 (1990).
9. P. E. Love *et al.*, *J. Exp. Med.* **179**, 1485 (1994).
10. B. A. Irving and A. Weiss, *Cell* **64**, 891 (1991); C. Romeo, M. Amiot, B. Seed, *ibid.* **68**, 889 (1992); F. Letourneur and R. D. Klausner, *Science* **255**, 79 (1992); B. A. Irving, A. C. Chan, A. Weiss, *J. Exp. Med.* **177**, 1093 (1993).
11. A. M. K. Wegener *et al.*, *Cell* **68**, 83 (1992).
12. D. R. Greaves, F. D. Wilson, G. Lang, D. Kioussis, *ibid.* **56**, 979 (1989).
13. E. W. Shores and P. E. Love, unpublished data.
14. H. Ohno *et al.*, *EMBO J.* **12**, 4357 (1993).
15. R. Testi, J. H. Phillips, L. L. Lanier, *J. Immunol.* **143**, 1123 (1989); W. Swat, M. Dessing, H. von Boehmer, P. Kieslow, *Eur. J. Immunol.* **23**, 739 (1993); D. Brandle, S. Muller, C. Muller, H. Pircher, *ibid.* **24**, 145 (1994).
16. L. A. Turka *et al.*, *Science* **253**, 778 (1991); P. Borgula, H. Kishi, Y. Uematsu, H. von Boehmer, *Cell* **69**, 529 (1992); D. Brandle, C. Muller, T. Ruclicke, H. Hengartner, P. Henspeter, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 9529 (1992).
17. Thymocytes (1×10^8) were treated with water-soluble Bolton-Hunter reagent (Pierce Chemical) and then radioiodinated with lactoperoxidase [J. A. Thompson, A. L. Lau, D. D. Cunningham, *Biochemistry* **26**, 743 (1987)]. Surface-labeled cells were solubilized at 4°C in lysis buffer (1% digitonin, 0.15 M NaCl, and 50 mM tris, pH 7.4), immunoprecipitated, analyzed by two-dimensional nonreducing (NR) (13%)–reducing (R) (14%) SDS-PAGE, and visualized by autoradiography (*9*). Rabbit antibodies 528 and 551 to ζ were generated to peptides that corresponded to amino acids 88 through 101 or 151 through 164 of murine ζ, respectively.
18. For multicolor FCM, we first incubated thymocytes or lymph node cells with antibody to the Fc receptor [monoclonal antibody (mAb) 2.4G2; J. C. Unkless, *J. Exp. Med.* **150**, 580 (1979)] to prevent nonspecific binding of conjugated mAbs. For two- and three-color FCM, cells were sequentially incubated with fluorescein isothiocyanate (FITC)–conjugated antibodies, phycoerythrin (PE)–conjugated antibodies, biotinylated antibodies, and then with Red 670 streptavidin (Gibco–Bethesda Research Laboratories). The FCM was done on a FACScan [Becton-Dickenson Immunocytometry Systems (BDIS)] with standard Lysis II software or software designed by the Division of Computer Research and Technology at the National Institutes of Health. Data were collected on 2×10^5 viable cells as determined by forward and side-light scatter. The mAbs used for FCM analysis included FITC conjugated to antibody to Leu 4, PE conjugated to antibody to Leu 4, and biotin conjugated to antibody to Leu 4 (BDIS); FITC conjugated to mouse IgG2a control antibody, PE conjugated to mouse IgG2a control antibody, FITC conjugated to antibody to CD4 (RM4.5), FITC conjugated to antibody to TCRβ (H57-597), PE conjugated to antibody to CD8 (53.67), and FITC conjugated to antibody to CD69 (H1.2F3) (Pharmingen).
19. T. Nakayama, A. Singer, E. D. Hsi, L. E. Samelson, *Nature* **341**, 651 (1989).

20. We thank A. M. Weissman and D. L. Weist for providing antibodies to ζ; R. D. Klausner, A. Rosenberg, L. E. Samelson, M. Vacchio, and A. M. Weissman for reading of the manuscript; and A. Singer for discussion and review of the manuscript.

27 June 1994; accepted 14 September 1994