The EMBO Journal vol.11 no.7 pp.2735–2746, 1992

T cell receptor (TCR) β chain homodimers on the surface of immature but not mature α, γ, δ chain deficient T cell lines

Marcus Groettrup, Agnes Baron, Gillian Griffiths, Ronald Palacios and Harald von Boehmer

Basel Institute for Immunology, Grenzacherstr 487, Postfach, CH-4005 Basel, Switzerland

Communicated by H.von Boehmer

Transfected T cell receptor (TCR) β chain genes are expressed as homodimers on the surface of immature (Sci/ET27F) but not on mature ($58\alpha^-\beta^-$) T cell lines which lack TCR α, γ and δ chains. The homodimer on Sci/ET27F cells is tightly bound to CD3δ and CD3ϵ while the association with CD3γ and CD3ζ proteins is rather weak. Crosslinking of the TCR β homodimers resulted in a strong and rapid calcium flux. In $58\alpha^-\beta^-$ T cells the β TCR chain could be easily visualized intracellularly but was not transported to the cell surface. The Scid cell lines considerably facilitate the molecular analysis of early differentiation events in the thymus which are likely to be regulated by the β TCR homodimer.

Key words: $\alpha\beta$ T cell receptor/CD3 complex/immature T cells/β TCR homodimer

Introduction

The $\alpha\beta$ T cell receptor is translated from genes which result from rearrangement processes taking place in a temporal order during T cell development. Rearrangement occurs first at the β TCR locus and the β TCR chain is formed before the α TCR chain: rearrangements and mRNA for the TCR β chain were detected in thymocytes on day 15 of fetal development, but no message for TCR α chain could be detected before day 16 (Raulet *et al.*, 1985; Snodgrass *et al.*, 1985; Haars *et al.*, 1986). We know very little about the regulatory role of the β TCR protein in T cell development. With regard to allelic exclusion of the β TCR locus it was shown that mice with a functional TCR β transgene do not rearrange endogenous TCR V_β gene segments (Uematsu *et al.*, 1988). This suppression requires the β TCR protein since introduction of a frame shift mutation abolishes suppression of endogenous β chain rearrangement (Krimpenfort *et al.*, 1989). It is unknown in which form and by what mechanism a β TCR protein mediates allelic exclusion.

From studies of comparable events during B cell maturation it is known that the Ig heavy chain can be expressed on the surface of pre-B cells in association with a so-called 'surrogate light chain' (Pillai and Baltimore, 1987, 1988; Tsubata and Reth, 1990) consisting of the Vpre-B and $\lambda5$ gene products (Sakaguchi and Melchers, 1986; Kudo and Melchers, 1987) before a light chain is rearranged. It is a subject of current discussion, whether or not the membrane expression of Ig heavy chain together with surrogate light chain at this stage of B cell development is involved in allelic exclusion of the heavy chain locus and whether it can induce rearrangement of the Ig light chain locus (Nussenzweig *et al.*, 1987; Reth *et al.*, 1987; Manz *et al.*, 1988; Kitamura *et al.*, 1991).

Transport of the $\alpha\beta$ T cell receptor to the cell surface normally requires the presence of an octameric minimal complex consisting of the disulfide-linked TCR $\alpha\beta$ heterodimer, the CD3ζ_2 or CD3$\zeta\eta$ dimer, two CD3ϵ chains and a single copy of CD3γ and CD3δ proteins (for review see Ashwell and Klausner, 1990). Studies with a mutant murine T cell line revealed that the deficiency of a single chain of the CD3 complex (CD3δ) resulted in retention and degradation of the entire complex in the endoplasmic reticulum (Bonifacino *et al.*, 1989). In murine and human mature T cell lines which lack the α TCR chain, the β TCR chain is neither expressed on the cell surface nor does it acquire resistance to Endo H in the Golgi system (Yagüe *et al.*, 1985; Alarcon *et al.*, 1988).

The same is apparently not true for immature T cells: it was shown that in immature $CD4^-8^-$ thymocytes of β TCR chain transgenic mice the TCR β chain is expressed on the surface but not linked to TCR α or γ chains (von Boehmer *et al.*, 1988). Surface expression of TCR β chain in the absence of other TCR chains was also documented in immature $CD4^-8^-$ and $CD4^+8^+$ thymocytes of β TCR transgenic Scid mice (Kishi *et al.*, 1991). In these studies the biochemical characterization of the surface expressed TCR β chain was impeded by the varying number of fragile thymocytes which can be obtained from these mice. In order to have an abundant source of biological material for biochemical characterization and to compare directly the fate of β TCR proteins in immature and mature T cells, we transfected productive β TCR genes into two cell lines which do not produce TCR α, γ and δ proteins, namely the mature T cell hybridoma $58\alpha^-\beta^-$ and the Scid mouse derived, immature cell line Sci/ET27F. Here we report the analysis of these cell lines before and after transfection and discuss the implication of these data with regard to T cell development.

Results

Characterization of the pre-T cell line Sci/ET27F with cell surface staining before and after transfection of a TCR β chain

The Sci/ET27F cell line was obtained from the thymus of C.B.-17 Scid mice and was established in culture as detailed in Materials and methods. The phenotype of Sci/ET27F cells, which grow continuously in culture in the absence of added growth factors, is $Thy1^+$, $CD3\epsilon^-$, $CD4^-$, $CD8^{-/lo}$, $TCR\beta^-$, TCR-$V\beta8^-$, Mac-1^-, $B220^-$, Pgp-1^-, $CD2^{-/lo}$, IL-$2R\alpha^+$, HSA^+ (Figure 1) and resembles that of a pre-T

© Oxford University Press

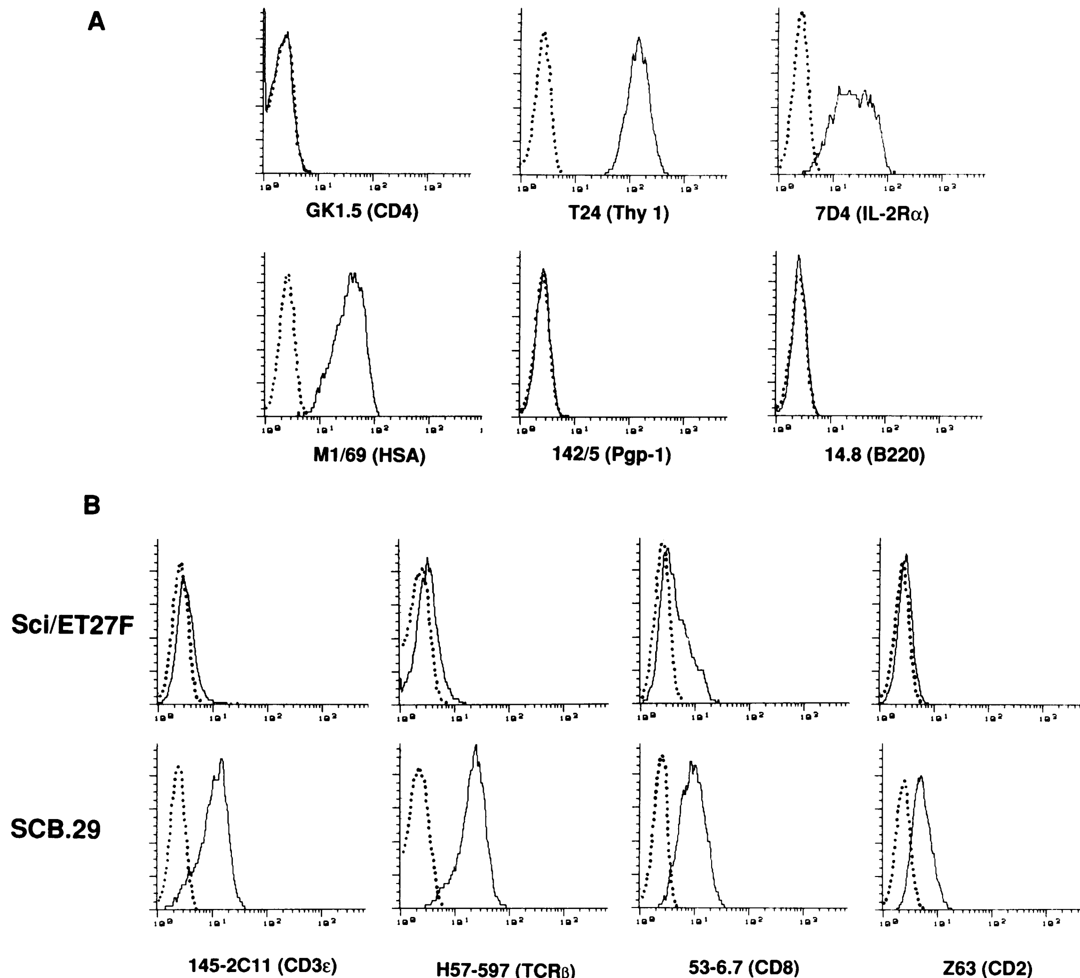

Fig. 1. Flow cytometric analysis of Sci/ET27F and SCB.29. Logarithmic fluorescence after one colour staining with indicated mAb is plotted versus cell number. (**A**) Sci/ET27F stained with markers which did not alter the expression pattern after transfection with TCR β. (**B**) Staining of Sci/ET27F and its transfectant SCB.29 with markers, the expression pattern of which changed after TCR β chain transfection.

cell. For that reason we refer to the cell line as pre-T cell line Sci/ET27F. Since productive TCR rearrangements occur infrequently in Scid mice (Schuler *et al.*, 1986), the development of thymocytes is apparently arrested at a stage before the TCR genes are rearranged corresponding to the phenotype of thymocytes from around day 14–15 of gestation.

This cell line was stably transfected by electroporation with a genomic clone bearing a functionally rearranged TCR β chain, HYβ9-1.14-5 (pTCF), which is derived from the male specific D^b restricted T cell clone B6.2.16, the same clone which had been used for microinjection to obtain TCR β transgenic mice (Uematsu *et al.*, 1988). This TCR β gene is composed of the J_β 2.3 and V_β 8.2 segments and the protein can therefore be immunoprecipitated and stained with the $V_\beta 8$ reactive monoclonal antibody F23.1 (Staerz *et al.*, 1985). Out of 35 analyzed neomycin resistant clones, 19 had become $CD3\epsilon^+$, TCR β^+, F23.1$^+$ and were designated SCB.6, SCB.24, SCB.29 and so on (Figure 1B). Comparison of the untransfected cell line and two transfected clones by the expression of surface markers from the panel mentioned above, showed that expression of the TCR β chain and CD3ϵ also led to an increase of CD2 and a slight increase of CD8 proteins.

To rule out that this result was dependent on a unique β TCR chain, we repeated the transfection experiment using another TCR β genomic clone (pK2.2) which was derived from an influenza virus hemagglutinin reactive T cell clone V2.1 (Taylor *et al.*, 1990), which also contains the $V_\beta 8.2$ segment but a different J segment ($J_\beta 2.1$) as well as different junctional sequences. Again 15 out of 20 neomycin resistant clones were scored as F23.1$^+$ and CD3$^+$.

Northern blot analysis of TCR and CD3 gene expression in Sci/ET27F and its transfectants

Total cellular RNA was isolated from the untransfected cell line Sci/ET27F and for most analyses from two representative TCR-β surface positive clones SCB.24 and SCB.29 as well as from one surface negative neomycin resistant clone SCB.32. Hybridization with a radiolabeled cDNA probe encoding the TCR$-C_\alpha$ region readily detected 1.7 kb full length message in the B6.2.16BW positive control, a hybridoma of the B6.2.16 T cell clone from which the β TCR gene was derived. In contrast, even after 3 weeks of exposure no TCR α transcripts were visible in Sci/ET27F and its βTCR gene transfectants (Figure 2A). Using a $V_\beta 8$ cDNA probe, full length message (1.3 kb) of the transfected TCR β chain gene was detected in greater abundance in the two surface positive transfectants, in a smaller quantity in the B6.2.16BW positive control, but not

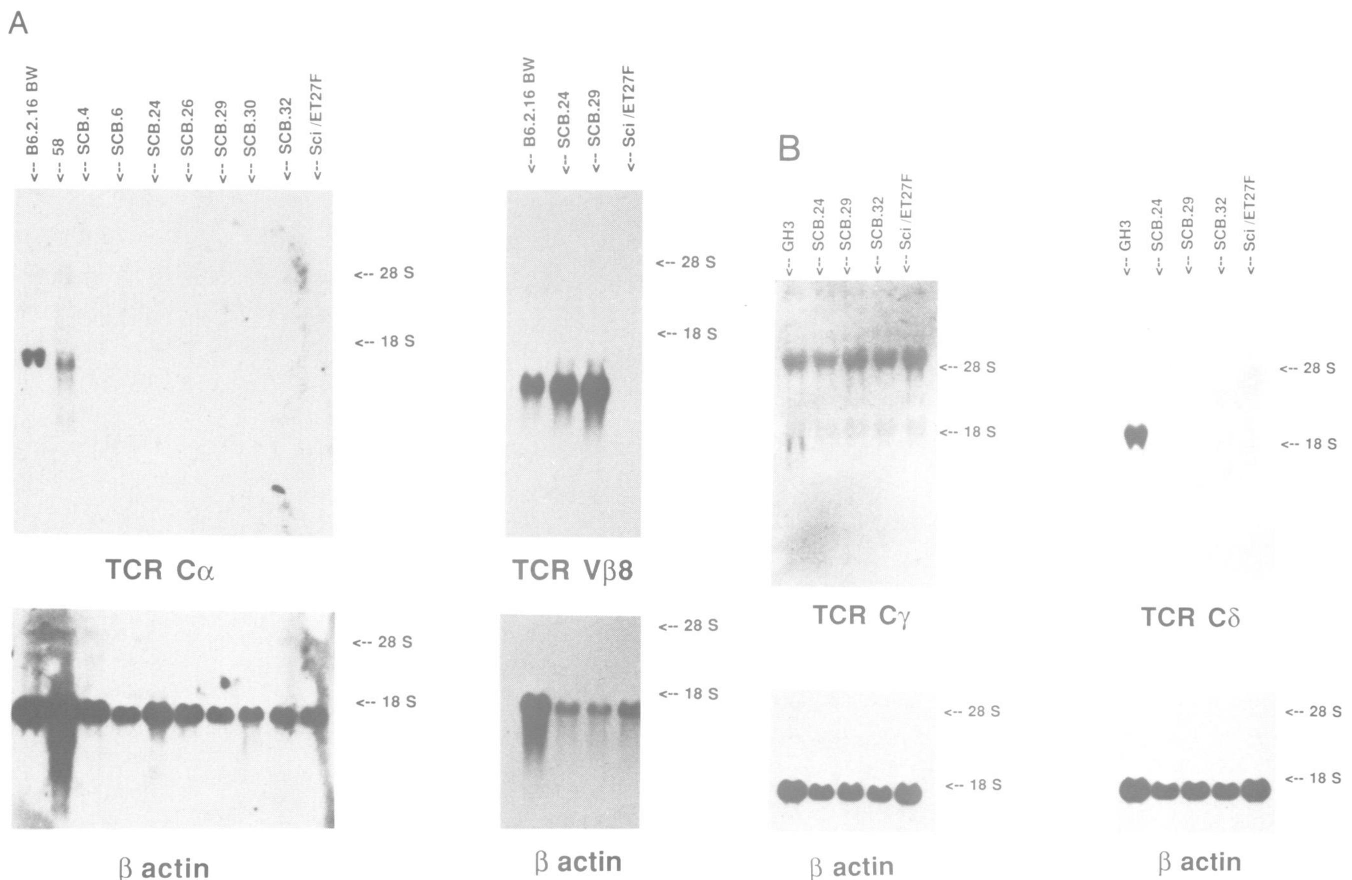

Fig. 2. Northern analysis of TCR transcription of Sci/ET27F and its transfectants. After hybridization of indicated probes (below the blot), blots were stripped and rehybridized with a β-actin probe. The source of total RNA is indicated above each lane. (**A**) Transcription of TCR α chain (1.7 kb) and of the transfected TCR β chain (1.3 kb); pos. control: B6.2.16BW (and $58\alpha^-\beta^-$, 58). (**B**) Transcription of TCR γ (1.5 kb, below 18S rRNA (=1.9 kb)) and TCR δ (2.0 kb) chains; pos. control: GH3.

in the untransfected cell line. As shown in Figure 2B, no γ or δ TCR transcripts were detected in Sci/ET27F and the SCB clones with γ or δ constant region probes, while the same probes hybridized to RNA in the γδ T cell hybridoma GH3 (G.Sim, unpublished). Other blots were hybridized with cDNA probes specific for the CD3γ, -δ, -ϵ and -ζ genes and message of all CD3 genes was found in Sci/ET27F, SCB24, SCB29 and SCB32 clones in similar amounts and of the same size as in the B6.2.16BW control (data not shown).

Surface expression of a TCR-β homodimer in the absence of TCR α, γ and δ proteins on immature T cells

The data obtained by surface staining and Northern blot analysis were confirmed by immunoprecipitation. The Sci/ET27F cell line, SCB clones, as well as hybridomas B6.2.16BW (TCR αβ) and GH3 (TCR γδ) as positive controls were surface iodinated and lysed in a 2% Triton X-100 lysis buffer. Immunoprecipitations were performed with antibodies against constant regions of TCR α, γ and δ as well as with the F23.1 antibody and precipitates were analyzed by 5−12% gradient PAGE under reducing and non-reducing conditions followed by autoradiography (Figure 3A). The same volume of lysate and the same amount of radioactivity was used for each immunoprecipitation; we lysed twice as many cells from SCB clones and Sci/ET27F as cells from positive controls.

The F23.1 antibody precipitated from SCB24 and SCB29 proteins of 80 and 40 kDa under non-reducing and reducing conditions respectively, while no such proteins were precipitated from Sci/ET27F and the surface TCR β negative transfectant SCB.32. With a TCR$-C_\alpha$ rabbit antiserum we precipitated the TCR αβ heterodimer from B6.2.16BW but no material from the SCB clones. Thus, the TCR β chain on SCB.24 and SCB.29 is disulfide-linked to a protein of the same mol. wt which is not a TCR α chain.

It was shown that in the absence of an α TCR chain, the TCR β chain can pair with a TCR δ chain (Hochstenbach and Brenner, 1989). In order to rule out, that on the SCB clones the TCR β chain is disulfide-linked to TCR γ or TCR δ chains, precipitations were performed with monoclonal antibodies KN365 and 3A10. The TCR γδ heterodimer was precipitated from the γδ hybridoma GH3 with both antibodies under non-reducing conditions, whereas no material was precipitated from the SCB24 and SCB29 clones.

Two dimensional non-equilibrium pH gradient electrophoresis (NEPHGE)/PAGE analysis was performed on reduced and non-reduced F23.1 precipitates from SCB29 and B6.2.16BW (Figure 4A). While B6.2.16BW yielded the expected pattern of an 80 kDa band at neutral pI which separated after reduction into the 40 kDa acidic TCR α and basic TCR β chains, only one band each in the non-reduced

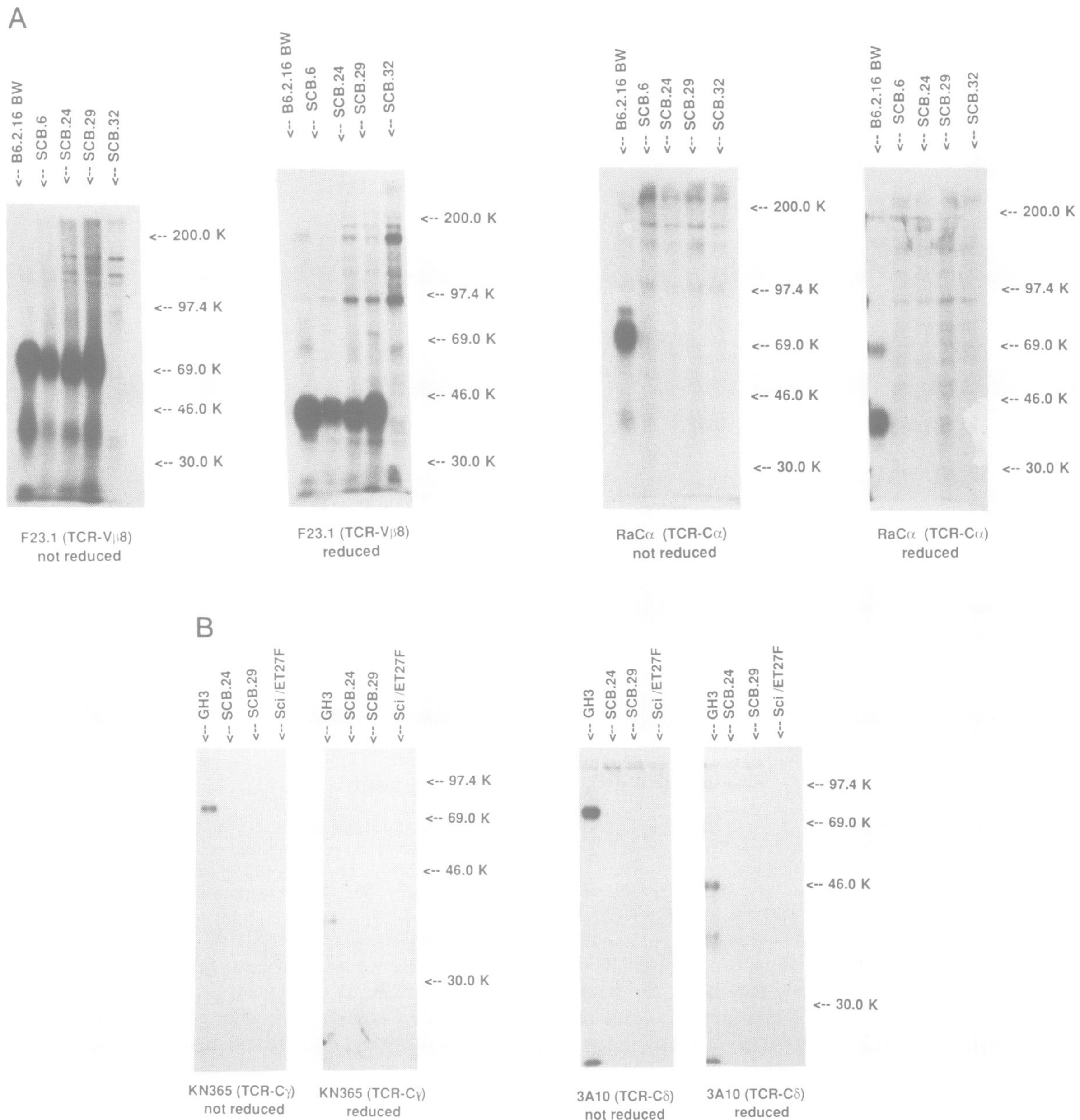

Fig. 3. Immunoprecipitation of TCRs from Triton X-100 lysates of surface iodinated cells (indicated above each lane). Antibodies used for immunoprecipitation (ipp) and reduction state are indicated below each panel. (**A**) Ipp with mAb F23.1 and rabbit serum anti-TCR$-C_\alpha$; pos. control: B6.2.16BW. (**B**) Ipp with mAb 3A10 and KN365; pos. control: GH3, KN365 demands denaturation of proteins in lsyates [1% SDS, heating for 5 min at 68°C, supplementation with 20 mM iodoacetamide, dilution 1:5 with TBS (150 mM NaCl, 1.5% Triton X-100, 10 mM Tris−HCl pH 8)] before ipp. Reduction was done before ipp therefore TCR δ (42 K) is not coprecipitated with TCRγ (37 K).

(80 kDa) and the reduced (40 kDa) gel, was detected with SCB.29 cells, which both appear to have the same charge in the NEPHGE dimension. Both bands were run to the very same position that the β chain of B6.1.16BW migrated to. Hence, the TCR β chain exists in disulfide linkage to a protein of the same mol. wt and the same charge, most likely as a homodimer. The striped pattern of the reduced B6.2.16BW β chain is very similar to that of the SCB.29 precipitate indicating a similar glycosylation with charged sugars. To further substantiate the surface expression of a β TCR homodimer, we cut out the B6.2.16BW TCR α and β bands as well as the SCB.29 band from the reduced gels and analyzed these three bands after partial proteolytic digestion with *Staphylococcus* V8 protease in the same gel (Figure 4B). The SCB.29 band gave the same pattern as the TCR-β chain itself and was quite distinct from the pattern of the TCR-α chain; taken together, these data strongly suggest that the TCR β chain on the immature cell line SCB.29 is expressed on the surface as a disulfide-linked homodimer.

Coimmunoprecipitations of the TCR β homodimer with CD3 proteins reveal tight linkage to CD3δ and CD3ε, but unusually loose association with CD3γ and CD3ζ

Surface iodinated B6.2.16BW, Sci/ET27F and SCB clones were lysed with a 1% digitonin/0.12% Triton X-100 lysis buffer and immunoprecipitations were performed with

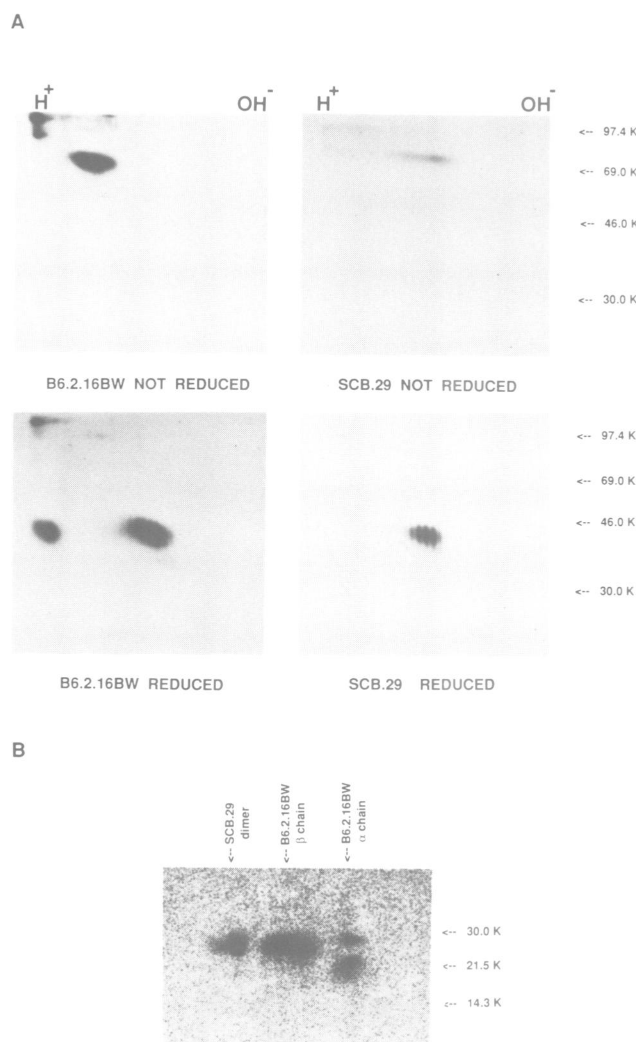

Fig. 4. Two-dimensional NEPHGE/PAGE analysis and V8 protease mapping of F23.1 immunoprecipitates from surface iodinated B.6.1.16BW and SCB.29 cells. (**A**) Both dimensions are run either not reduced (upper panels) or reduced (lower panels). Acidic and basic ends of the NEPHGE gel rod are indicated (H^+, OH^-). Four-fold less SCB.29 non-reduced than reduced material was loaded to avoid non-specific precipitation in the rod. (**B**) From the reduced 2D gels shown, B.6.2.16BW acidic α and basic β chain as well as the SCB.29 band were cut out, swollen in sample buffer with 1% agarose in the gel, topped with 2 ml of 1 mg/ml V8 protease, digested during migration at 20 V for 9 h followed by separation of fragments on 15% discontinuous PAGE.

monoclonal antibodies against CD3γ (HMT3.2) and CD3ε (500A2) as well as with rabbit antisera reactive with CD3δ (J31) and CD3ζ (N39). As a control we precipitated the Thy1 (mAb T24) molecule, which is known to be present on the surface of all these cells in similar amounts. Precipitates were subsequently analyzed on reduced and non-reduced 7.5–17% PAGE gradient gels (Figure 5A). All four CD3 antibodies coprecipitated the TCR αβ heterodimer from B6.2.16BW lysates. Surprisingly, only the antibodies reactive with CD3δ and CD3ε but not those reactive with CD3γ and CD3ζ precipitated the TCR β homodimer from SCB.24 and SCB.29 under experimental conditions which normally leave associations with the TCR αβ–CD3 octameric complex undisrupted. These results have been confirmed by performing immunoprecipitations with different rabbit antisera against CD3γ, CD3δ and CD3ζ chains (Kishi *et al.*, 1991). Coimmunoprecipitation of the TCR β homodimer with CD3γ and CD3ζ was detected only when no detergent other than 1% digitonin was used throughout the protocol (Figure 5B), indicating an unusually weak association. Further, it should be mentioned that the CD3ζ chain is precipitated not only from the cell surface of SCB clones, but also from the untransfected Sci/EF27F line as a 32 and a 16 kDa protein under non-reduced and reduced conditions respectively. Apparently the CD3ζ chain can reach the cell surface even in the absence of TCR β chain expression in these immature T cell lines. Nevertheless, so far we have been unable to detect any other proteins except CD3 being specifically associated with the TCR β homodimer.

Crosslinking of TCR β homodimers on immature T cells results in receptor downmodulation and intracellular calcium flux

Thymocytes from αβ TCR transgenic mice which express α and β TCR chains from the B6.2.16 T cell clone, the Sci/ET27F thymocyte cell line and its transfectant SCB29 were loaded with the calcium chelating fluorescent dye indo-1AM. The cells were treated with monoclonal antibodies F23.1 ($V_\beta 8$), 500A2 (CD3ε) and T3.70 (B6.2.16 TCR αβ clonotype; Teh *et al.*, 1988). Twenty seconds later these antibodies were crosslinked with a large excess of goat anti-mouse Ig antiserum and the intracellular calcium concentration was measured by monitoring the fluorescence emission at 405 and 525 nm over 12 min (Figure 6). Crosslinking with F23.1 and 500A2 resulted in a very rapid, vigorous and transient rise in the cytoplasmic calcium concentration of SCB.29. The kinetics of the flux were similar for both antibodies and distinct in onset and shape from that of the bulk thymocytes, which were used as a control. A lag phase of ~2 min between crosslinking and start of the flux was seen in bulk thymocytes, but not in SCB.29 cells. The T3.70 antibody does not trigger the TCR β homodimer but only the thymocytes, which bear the B6.2.16 T cell receptor; this was expected, since the T3.70 epitope depends upon the presence of the clonotypic TCR α chain.

In spite of the signal transmission by the β TCR homodimer we could not detect any changes in viability and proliferation when the SCB.29 cells were cultured in plastic dishes coated with F23.1 and analyzed after 15 and 60 h of incubation. Flow cytometric analysis with the panel of antibodies mentioned in Figure 1 revealed that the only difference to cells cultured in the absence of F23.1 antibodies was reduced staining with F23.1 and CD3ε antibodies as well as an upregulation of the IL-2 receptor level, indicating activation of the T cells (data not shown).

Mature T cells which lack the TCR α chain do not express the TCR β chains on the surface but retain the β protein intracellularly

The mature T cell hybridoma $58\alpha^-\beta^-$ (Letourneur and Malissen, 1989) has been selected such that it does not express functional TCR α and β chains, but still expresses all proteins of the CD3 complex. After transfecting this cell line with the genomic clone of the B6.2.16 TCR β chain HYβ9-1,14-5 (pTCF) by electroporation, none of the 47 analyzed neomycin resistant clones (named $58\alpha^-\beta y$) became $F23.1^+$ or $500A2^+$ as determined by surface

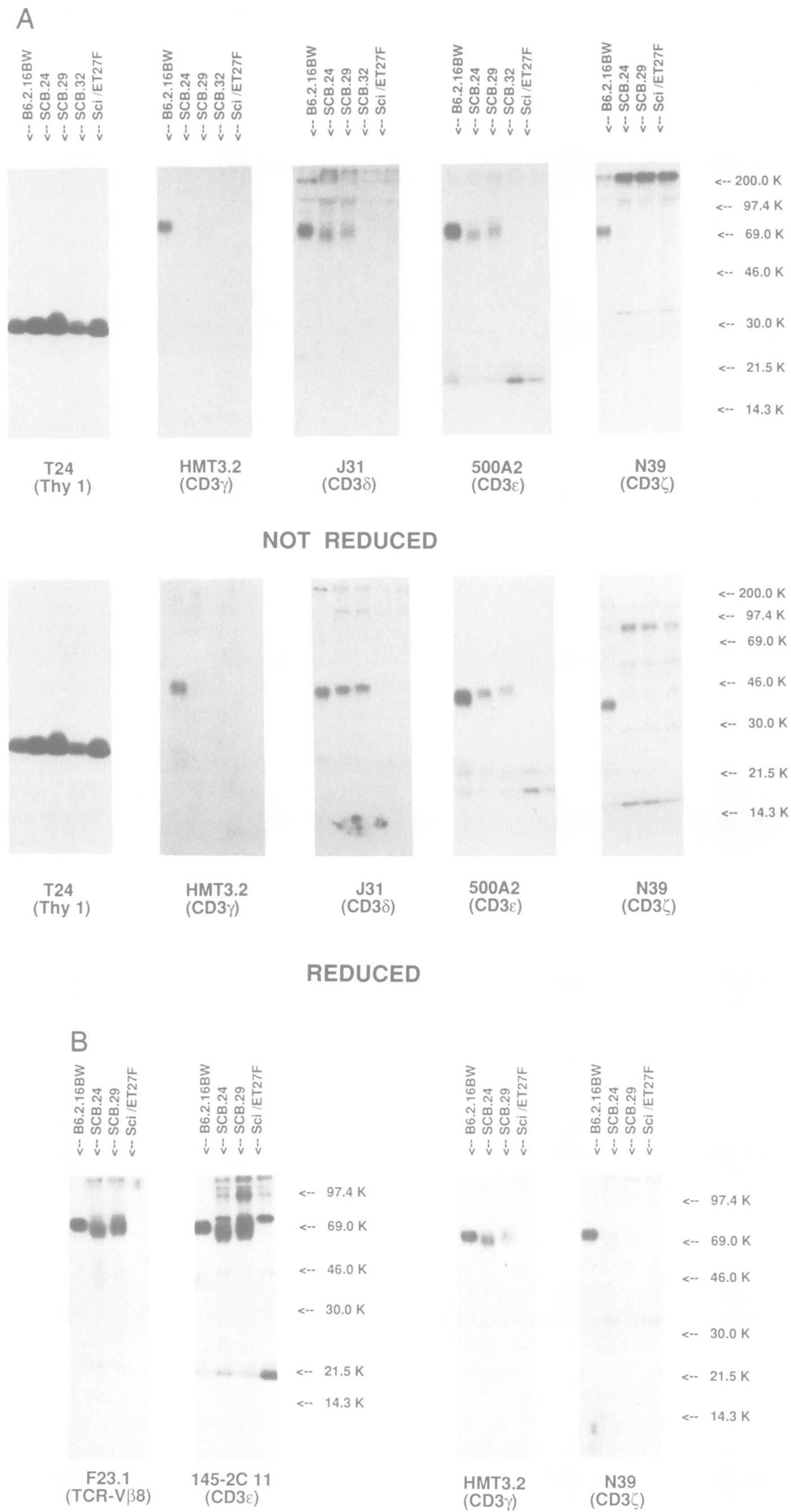

Fig. 5. Immunoprecipitation from lysates of surface iodinated cells with anti-CD3 antibodies. (**A**) Cells (indicated above each lane) were lysed with 1% digitonin/0.12% Triton X-100 lysis buffer and precipitates were washed as detailed in Materials and methods. Coimmunoprecipitation of TCR dimers in unreduced (80 K, upper panels) and reduced (40 K, lower panels) gel with antibodies indicated below each panel. Mol. wts of murine CD3:γ (21 K), δ (26 K), ϵ (25 K), ζ_2 (32 K). All bands above 97.4 K and in the 500A2 panels below 21.5 K cannot be assigned. (**B**) Cells were lysed with 1% digitonin lysis buffer and precipitates were washed 4-fold with the lysis buffer. All gels shown are unreduced.

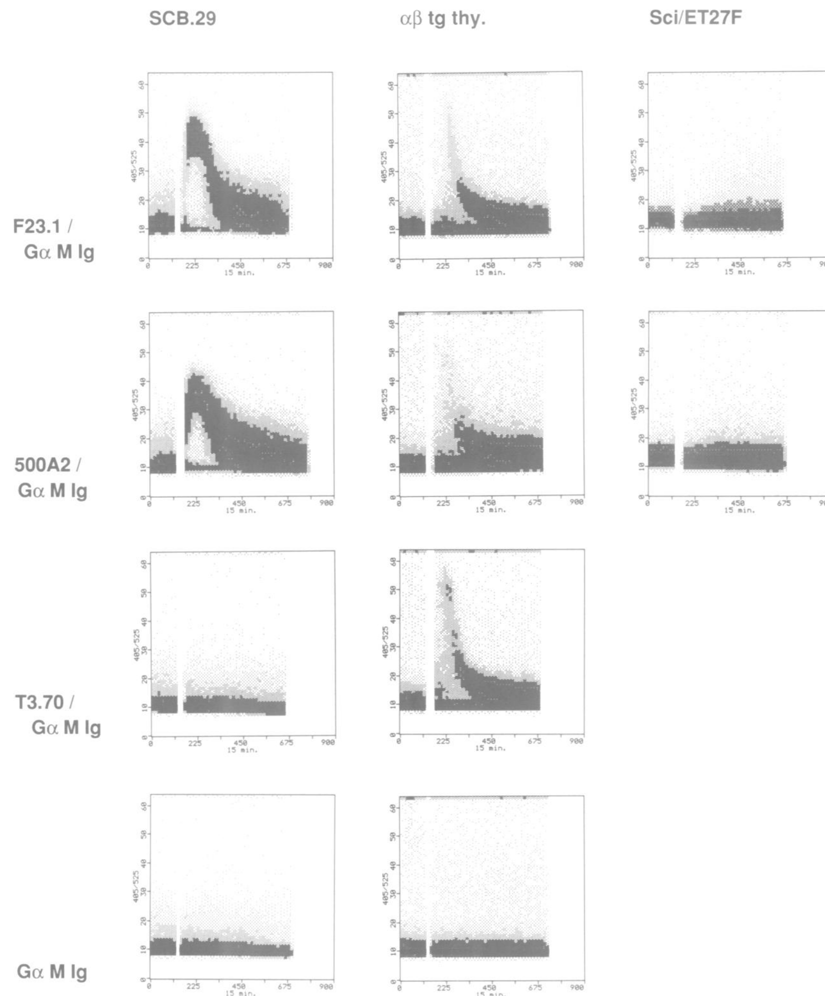

Fig. 6. Calcium flux in TCR $\alpha\beta$ transgenic thymocytes, Sci/ET27F and SCB.29 (indicated above) after triggering with mAbs F23.1, 500A2 and T3.70 (indicated left) and crosslinking with goat anti-mouse Ig antiserum (GαM Ig). The emission ratio is plotted (405 nm/525 nm) versus time (15 min). Negative control: GαM Ig only.

staining and immunoprecipitation. Even cultivation of two clones for 2 months and subsequent cell sorting did not yield any cells expressing the TCR β chain on the surface. Northern blot analysis of these two transfectants demonstrated for each clone as much full length message for the transfected TCR β chain as detected in SCB.24 or SCB.29 (Figure 7). We then cotransfected this TCR β chain gene together with a genomic clone (pTα215βen1) encoding a functional TCR α chain which was derived from the influenza virus hemagglutinin reactive T cell clone V2.1 (Taylor *et al.*, 1990). Out of 20 analyzed neomycin resistant clones (called 58αiβy) eight became F23.1$^+$ and 500A2$^+$, suggesting that in contrast to the Sci/ET27F pre-T cell line, this mature T cell line requires TCR α chains for transport of the TCR β chain to the cell surface.

In further studies five representative clones [58$\alpha^-\beta^-$ (mature untransfected), 58$\alpha^-\beta$y.1 (mature/TCR β only), 58αiβy.10 (mature/TCR $\alpha\beta$), Sci/ET27F (pre-T/untransfected) and SCB.29 (pre-T/TCR β only)] were tested for intracellular and surface β TCR chains by staining with F23.1 and analyzing protein expression by confocal microscopy as well as flow cytometry (Figure 8). The 58$\alpha^-\beta$y.1 clone expresses the TCR β protein intracellularly but not on the cell surface. The homogeneous cytoplasmic

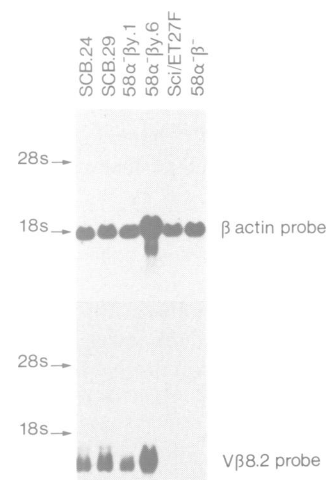

Fig. 7. Northern analysis of the amount of mRNA transcribed from a transfected TCR β chain gene in immature (SCB.24 and SCB.29) and mature (58α-βy.1, 58α-βy.6) T cell transfectants. Total RNA from cell lines indicated above each lane was blotted and hybridized with a $V_\beta 8.2$ probe and after removal of the probe rehybridized with a β-actin probe.

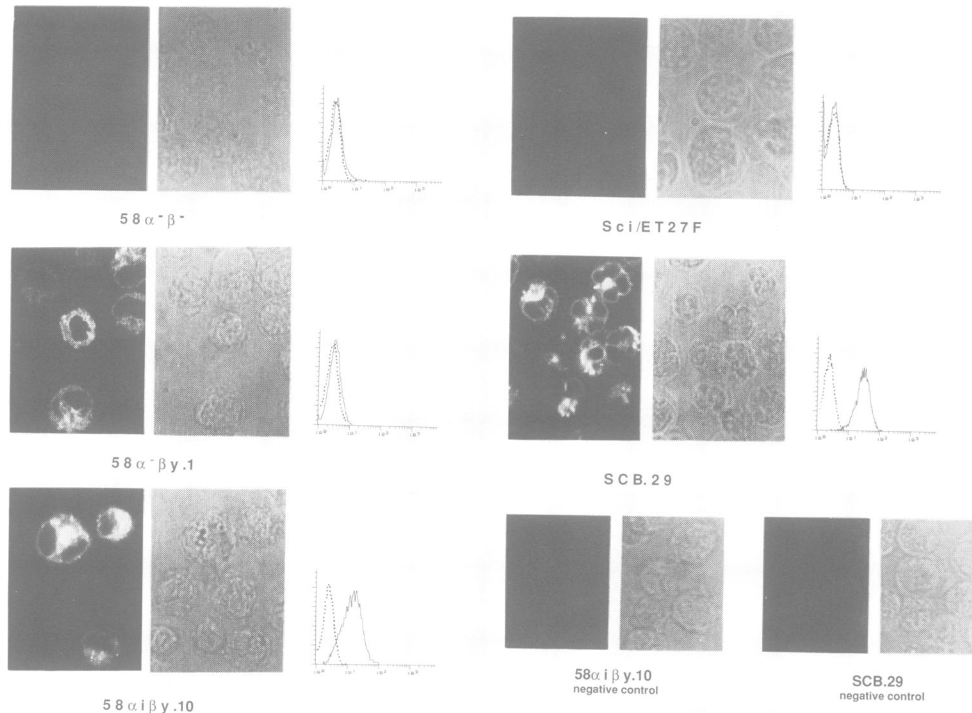

Fig. 8. Intracellular and surface staining with F23.1 of mature and immature T cells before and after transfection. Fluorescence and phase pictures from confocal microscopy (left) and flow cytometric analysis (right) of the same cell lines (indicated below) are shown. Negative control for intracellular staining: 58αiβy.10 and SCB.29 with second stage RαM Ig-FITC only.

and perinuclear staining is consistent with localization within the endoplasmic reticulum. In contrast, 58αiβy.10 and SCB.29, express the protein both intracellularly and on the cell surface and in both of these cells a bright localized cytoplasmic staining, as predicted by migration of the protein from the endoplasmic reticulum through the Golgi system to the cell surface, is observed. This is supported by former biochemical analysis of TCR α deficient mature T cells which showed that the TCR β chain did not acquire Endo H resistance in the Golgi system (Alarcon *et al.*, 1988). In other experiments intracellular staining of the TCR α chain in TCR α-transfected fibroblasts revealed retention of the TCR α chain in the ER (Lippincott-Schwartz *et al.*, 1988). Thus, these experiments clearly point to a difference between mature and an immature T cell line in their ability to transport the TCR β chain to the cell surface in the absence of a TCR α chain.

Discussion

Isolated $CD4^-8^-$ immature thymocytes of TCR β transgenic mice and total thymocytes from TCR β transgenic Scid mouse are able to express the TCR β chain on the cell surface in the absence of other TCR chains (von Boehmer *et al.*, 1988; Kishi *et al.*, 1991). We confirmed this unusual property of immature T cells by transfecting a TCR β chain gene into the Scid thymocyte line Sci/ET27F and analyzing it at the clonal level: the TCR β chain is surface expressed as a disulfide-linked homodimer which is bound tightly to CD3δ and CD3ε but only loosely to CD3γ and CD3ζ chains.

In the *in vivo* models the TCR β chain is expressed on the surface in about equal portions as a disulfide-linked dimer and as a monomer. Immunoprecipitations with F23.1 also demonstrated that substantial amounts of the precipitate are disulfide bound in a poorly defined high mol. wt complex. We have tried to identify further the mol. wt of this complex with low percentage gradient gels; however, this material gave no distinct bands but rather a smear reaching from 100 kDa to the top of the gel. It is difficult to decide whether the macromolecular and the monomeric forms of the TCR β chain in these thymocytes have physiological relevance or whether they are artificially generated by iodinating highly fragile immature thymocytes.

Kishi and coworkers reported that the TCR β chain is expressed on the surface of thymocytes from TCR β tg Scid mice in the absence of any components of the CD3 complex as determined by surface staining and coimmunoprecipitation (Kishi *et al.*, 1991). Since this is in contradiction to coexpression of CD3 and TCR β chain in our Scid-derived cell lines, we repeated these experiments by staining thymocytes from several TCR β transgenic Scid mice with F23.1 and two monoclonal antibodies reactive with CD3ε chains (500A2 and 145-2C11). We found these thymocytes to be homogeneously positive for $F23.1^+$ and although the previously used 500A2 antibody stained them only weakly, the 145-2C11 antibody clearly showed CD3ε surface expression (Figure 9A). Further, we could coimmunoprecipitate the TCR β chain with the J31 anti-CD3δ antiserum. Thus, immature T cells express TCR β dimers on the cell surface together with CD3δ and CD3ε both *in vivo* and *in vitro*.

Recently a cell line (KKF) has been derived from a lymphoma of a Gross virus infected BALB/K mouse (Punt *et al.*, 1991). The KKF line does not express functional α, γ or δ TCR chains and also expresses a TCR β chain on the cell surface. The β chain in KKF is present as a monomer as well as a dimer, CD3ε antibodies coprecipitated the TCR β dimer, and also in this cell line association with CD3ζ

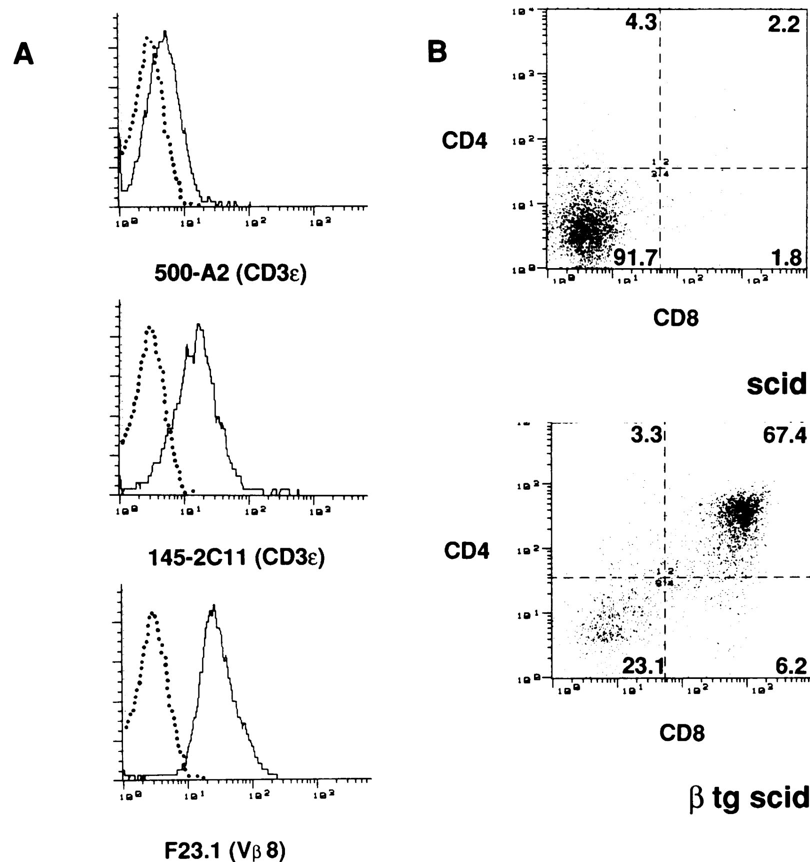

Fig. 9. Flow cytometric analysis of TCR β transgenic Scid thymocytes. (**A**) Staining of β Tg.Scid thymocytes with 500-A2, 145-2C11 and F23.1 as indicated. (**B**) CD4/CD8 double staining of thymocytes from TCR β transgenic Scid and non-transgenic C.B-17 Scid mice; the percentages of cells are indicated in each quadrant.

is weak. Compared with SCB.29, the KKF line has the advantage that it does not carry a transgene nor a transfected β chain, but it bears the caveat that retroviral recombinations might have altered the normal expression pattern. Furthermore this cell line makes the important point that expression of TCR β homodimers is not confined to $V_{\beta}8$ containing TCR β chains.

The composition of the TCR β/CD3 complex on SCB.29 cells may not be so unique, as indicated by three recent findings. First, a cell line has been characterized which expresses the $\alpha\beta$ TCR on the surface without the need for CD3γ (Pérez-Aciego *et al.*, 1991). Second, the CD3ζ homodimer can reach the cell surface on NK cells in the absence of TCR or further CD3 chains in association with other surface proteins (Lanier *et al.*, 1989). Third, signal transduction in SCB.29 is possible although the TCR β homodimer is not closely associated with the CD3ζ_2 chains. This finding is supported by recent evidence that signalling via the TCR can be mediated by a cluster of CD3 chains independent of the CD3ζ chain (Wegener *et al.*, 1992). If CD3 components which contain negatively charged amino acids in their transmembrane region facilitate the TCR $\alpha\beta$ dimerization by intramembrane non-covalent interactions with the TCR α chain (two positive charges) and TCR β chain [one positive charge; (Bonifacino *et al.*, 1991)], it would be reasonable that TCR β dimerization requires a different association with CD3 chains.

At present the evidence that TCR β expression regulates thymocyte differentiation is rather indirect: introduction of a TCR β chain gene into Scid mice reproducibly results in the induction of CD4 and CD8 coexpression (Figure 9B) whereas transfection of a β chain into Sci/ET27F results if anything, in only a slight upregulation of CD8, but not of CD4. Possibly the CD4 and CD8 surface expression on thymocytes from β TCR transgenic Scid mice does not result from a direct intracellular regulation as for instance transplantation of non-Scid bone marrow into Scid mice (Shores *et al.*, 1990) can also result in CD4 and CD8 expression of Scid thymocytes. Interestingly, transfection of the TCR β chain leads to an increase in CD2 (LFA-2) surface expression. CD2, which is involved in the non-specific enhancement of cell–cell adhesion in lymphocytes by binding to LFA-3 and is believed to stimulate thymocyte proliferation, is strongly upregulated on fetal thymocytes during days 15 and 16 of gestation (Duplay *et al.*, 1989; Sen *et al.*, 1989), the same time that first rearrangements and transcriptions of the TCR β chain are detected.

We now need to study the molecular mechanism which allows surface expression of β TCR homodimers on immature T cells by comparing mature and immature transfectants. For instance it could be that a certain protein, expressed exclusively in pre-T cells, prevents degradation in the ER or acts as a chaperone to lead TCR β chains to the surface. So far we have been unable to coprecipitate an equivalent molecule to the surrogate light chain in pre-B cells from the surface of SCB.29. We cannot be certain that surface expression of a TCR β homodimer plays a pivotal role in thymocyte maturation, allelic exclusion, or induction

of TCR β chain rearrangement. However, the TCR β homodimer is expressed on the surface of a pre-T cell line and does transmit a distinct signal into the cell upon triggering from the extracellular environment. Biologically it would make sense if the positive feedback signal indicating a functional TCR β chain rearrangement resulted from the successful expression of the protein on the cell surface.

Materials and methods

Establishment in culture of pre-T cell lines from thymus of C.B-17 Scid mice

Previously we have found that the ET cortical thymic epithelial line (Palacios *et al.*, 1989) together with exogenous rIL2 (200 U/ml), rIL7 (500 U/ml), and supernatant from the FLS4-1 fetal liver stromal line (called F, final conc. 10% v/v; Palacios and Samaridis, 1991) efficiently support the long-term growth in culture of pre-T cells isolated from the thymus of fetal (day 13–14) and adult normal mice (R.Palacios, unpublished results). We were able to establish five pre-T cell lines from the thymus of C.B-17 Scid (8–10 weeks old) with this protocol. Briefly, 5×10^4 Scid thymocytes suspended in 2 ml of conditioned culture medium (Iscove's modified Dulbecco's medium + 7.5% FCS + 2 mM L-glutamine + 5×10^{-5} M 2-mercaptoethanol + 50 μg/ml gentamicin + rIL2, rIL7 and F) were cultured on monolayers of ET cortical thymic epithelial cells (~50% confluency) in six-well Costar plates (cat. no. 3506) at 37°C for 5–7 days. The thymocytes from each well were harvested, washed and resuspended in conditioned culture medium and distributed in three to six freshly prepared Costar wells containing ET thymic epithelial cells, in a final volume of 1.5–2 ml per well. Every 4–6 days, the cells were harvested, washed and cultured in freshly prepared conditioned culture medium and Costar plates. Five thymocyte lines (called Sci/ET27F) showed stable and continuous growth in culture after 12 weeks of initiation of the cultures and were therefore considered established cell lines. After 6–7 months of culture, we found that the Sci/ET27F thymocyte lines could grow in culture medium without thymic epithelial cells and exogenous growth factors. The Sci/ET27F thymocyte line used here has been propagated in culture medium (without thymic epithelial cells and exogenous growth factors) by changing these cells into fresh culture medium every 3–4 days. Several aliquots of the Sci/ET27F lines were successfully frozen in IMDM + 20% FCS + 14% DMSO.

Transfection

Plasmids HYβ9-1.14-5 (pTCF) and pTα215βen1 were linearized and 30 μg DNA was dissolved in 600 μl PBS in which 6×10^6 cells were resuspended. Electroporation was performed in a Biorad Genepulser at 230 V and 960 μF. We selected transfectants in growth medium with 2 mg/ml (for $58\alpha^-\beta^-$) or 1.4 mg/ml (for Sci/ET27F) G418 (Gibco).

Flow cytometry

After washing once in PBS + 2% FCS (FM), 10^6 cells were stained in 100 μl with saturating concentrations of monoclonal antibodies (mAb) on ice for 15 min in 96 well plates. Cells were washed twice in 200 μl of FM and, if required, stained with a second stage reagent and washed again. Five thousand forward scatter/side scatter gated, alive cells were acquired and analyzed with a Becton Dickinson FACScan flowcytometer. Antibodies used: 1) FITC conjugated mAb: 142/5 (RαM Pgp-1, Trowbridge *et al.*, 1982), 145-2C11 (HαM Cd3ϵ, Leo *et al.*, 1987), 53-6.7 (RαM Lyt-2, Becton Dickinson), 7D4 (RαM IL-2Rα, Malek *et al.*, 1983). M1/69.16.11 (RαM HSA, Springer *et al.*, 1987), T24 (RαM Thy1, Dennert *et al.*, 1980). 2) PE conjugated mAb: GK1.5 (RαM CD4, Becton Dickinson). 3) Biotinylated mAb: F23.1 (MαM TCR Vb8.1 + 2 + 3, Staerz *et al.*, 1985), H57-597 (HαM TCR β, Kubo *et al.*, 1989). 4) Unconjugated mAb: 14.8 (RαM B220, Kincade *et al.*, 1981), 500A2 (HαM CD3ϵ, Havran *et al.*, 1987), M1/70.15 (RαM Mac-1, Boehringer), Z63 (RαM CD2, R.Rutschmann, manuscript in preparation). 5) Second stage reagents: SαM Ig F(ab')$_2$-FITC (Silenius) and Streptavidin-PE (Southern Biotechnology).

Northern blot analysis

Total RNA was prepared from cells with acid guanidinium thiocyanate phenol chloroform extraction as described (Chomczynski and Sacchi, 1987). RNA (15 μg/slot) was loaded and separated on 1.5% agarose–200 mM formaldehyde gels and RNA was blotted on Zeta Probe membranes (Biorad). Prehybridization (4 h) and hybridization (12 h) were performed in 1 mM EDTA, 0.5 M NaH_2PO_4, 7% SDS at 65°C, the cDNA probes were obtained with ^{32}P-random primed oligolabeling and applied at 2×10^6 c.p.m./ml. Washing procedures involved two steps in 1 mM EDTA, 40 mM NaH_2PO_4, 5% SDS and one step in 1 mM EDTA, 40 mM NaH_2PO_4, 1% SDS at 65°C for 30 min, each, followed by autoradiography. Blots were stripped by washing twice with 0.1 × SSC/0.5% SDS for 20 min at 95°C and rehybridized with a β-actin probe. Mouse cDNA fragments used for probing were: TCR–C_α (800 bp *Eco*RI–*Eco*RI from PαBDHI#1; T.Mak, Toronto), TCR–V_β8.2 (284 bp PCR fragment from B6.2.16BW β chain), TCR–C_γ (1600 bp *Bam*HI–*Bam*HI from pHDS4, S.Tonegawa; Cambridge, MA), TCR–C_δ (900 bp *Eco*RI–*Eco*RI from pCδ-11; W.Born, Denver, CO), CD3γ (750 bp *Hin*dIII–*Eco*RI from pB10AT3γ; M.Crumpton, London), CD3δ (640 bp *Eco*RI–*Eco*RI from pPEMT3δ; C.Terhorst, Boston, MA), CD3ϵ (1500 bp *Eco*RI–*Eco*RI from pDL1; C.Terhorst). CD3ζ (1200 bp *Eco*RI–*Eco*RI from pGEM3z; R.Klausner, Bethesda, MD), β-actin (110 bp *Pst*I–*Pst*I from pB-action; M.Wiles, Basel).

Surface iodination, immunoprecipitation, NEPHGE

Harvested cells were washed three times in PBS, 10^6–4×10^7 cells were resuspended in 1 ml PBS, and iodinated for 40 min at room temperature by addition of 50 μl 200 mM D-glucose, 10 μl of 100 mCi/ml Na^{125}I (Amersham), and 50 μl lactoperoxidase (50 U/ml, Sigma)/glucose oxidase (10 U/ml, Sigma) solution. After 4-fold washing in PBS/0.1% NaN_3, viability was determined by Trypan blue exclusion (>90% viable cells in each experiment). Lysis of the cells was performed—as all subsequent steps—on ice or at 4°C for 40 min in 1 ml of either [2% Triton X-100, 150 mM Nacl, 20 mM Tris–HCl pH 8, 1 mM $MgCl_2$, 10 mM iodoacetamide, 1 mM PMSF, 40 KIU/ml aprotinin (Boehringer), 5 μg/ml leupeptin (Boehringer)] or [1% digitonin, (±0.12% Triton X-100), 150 mM NaCl, 10 mM triethanolamine pH 7.8, 1 mM $MgCl_2$, 10 mM iodoacetamide, 1 mM PMSF, 40 KIU/ml aprotinin, 5 μg/ml leupeptin] lysis buffers and nuclei were removed by centrifugation for 10 min at 12 000 *g*. The radioactivity of lysates was determined. For immunoprecipitations (ipp) with rabbit antisera, aliquots of the lysate (2×10^6 cells ~50 μl ~10^7 c.p.m./ipp) were precleared for 1 h with 10 μl normal rabbit serum and two precipitations for 30 min with 20 μl protein A–sepharose CL-4B (pA) beads (Pharmacia), swollen in lysis buffer. Ipp was carried out for at least 2 h on ice and antibodies were precipitated with pA beads. Washing was performed twice, each time with 1 ml NET-TON buffer (650 mM NaCl, 5 mM EDTA, 50 mM Tris–HCl, 0.5% Triton X-100, 0.05% NaN_3, 1 mg/ml ovalbumin) and NET-T buffer (NET-TON with 0.15 M NaC and without ovalbumin). Proteins were eluted from beads with non-reduced or reduced sample buffer (10% glycerol, 2.3% SDS, 0.0625 M Tris–HCl pH 6.8, 0.05% w/v bromphenolblue, ±20 mM DTT) by boiling for 5 min and subsequent addition of iodoacetamide to a final concentration of 60 mM. Samples were run on discontinuous Laemmli PAGE. Gels were fixed, incubated in Enlightening^{TM} (NEN), dried and analyzed using a phosphoimager and by autoradiography. NEPHGE was performed as described (O'Farrell *et al.* 1977) for 1600 Vh, using the ampholyte Pharmalyte 2D 3-10 (Pharmacia). Antibodies used are: 500A2 (pA purified mAb, 1.96 mg/ml, 0.7 μl/ipp), 145-2C11 (pA purified mAb, 2.1 mg/ml, 1 μl/ipp), F23.1 (pA purified mAb, 1.3 mg/ml, 1 μl/ipp), T24 (pG purified mAb, 0.36 mg/ml, 4 μl/ipp), KN365 (supernatant for precoating of pA beads, MαM TCR-Cγ1 and C_γ2 recombinant protein, Bonneville *et al.*, 1988), 3A10 (supernatant for precoating of pA beads, HαM TCR–Cδ, Itohara *et al.*, 1989), RαCα (pA purified rabbit antiserum against murine TCR–Cα chimeric protein, Traunecker *et al.*, 1986), HMT3.2 (Hα human CD3γ, crossreactive with murine CD3γ, R.Kubo, Denver, CO; unpublished), J31 (rabbit antiserum against last 13 C-terminal amino acids of human CD3δ, crossreactive with murine CD3δ, 6 μl/ipp (Pérez-Aciego *et al.*, 1991), N39 (rabbit antiserum against peptide from human CD3ζ, crossreactive with murine CD3ζ, 6 μl/ipp, J.Sancho and C.Terhorst, unpublished), numbers 125, R9 and 390 are rabbit antisera reactive with murine CD3γ, CD3δ and CD3ζ respectively (Manolios *et al.*, 1991).

Intracellular calcium measurement

Thymocytes or cultured cells were resuspended at 10^7/ml in RPMI + 2% FCS + 5 μM indo-1AM (Calbiochem) and rotated in 5 ml polyallomer tube at 37°C for 45 min. Cells were washed twice and taken up in RPMI + 2% FCS at 10^6/ml. Triggering was done at 37°C by addition of purified monoclonal antibodies (F23.1, 500A2, T3.70) at the same concentration as that used for surface stainings to 10^5 cells with addition of 10 μl of GaM Ig antiserum (Jackson Immunoresearch labs) 20 s later. Calcium flux was monitored for 15 min on a Coulter Elite flow cytometer by measuring emission at 405 and 525 nm.

Confocal microscopy

Cells were fixed in 2% paraformaldehyde/PBS for 1 h at room temperature. Fixation was quenched by washing in 50 mM ammonium chloride/PBS and cells were permeabilized in PBS/0.01% saponin/0.25% gelatin/0.1% NP40.

Non-specific binding was blocked by incubation in 10% FCS for 15 min prior to addition of F23.1 for 30 min at room temperature. Samples were washed extensively in PBS/0.01% saponin/0.25% gelatin before incubation in second stage antiserum (rabbit αM Ig, FITC, DAKOPATTS). Samples were washed as before, mounted in PBS/90% glycerol containing 2.5% DABCO and analyzed using a Biorad 600 confocal microscope.

Acknowledgements

We thank Katrin Hafen, Mark Dessing and Silke Hennecke for excellent technical instruction and help. Generous antibody gifts were obtained from C.Terhorst (HMT3.2, J31 and N39), R.Klausner (no. 125, R9 and no. 390), S.Tonegawa (KN365 and 3A10) and A.Traunecker (RαCα). K.Karjalainen is acknowledged for contributing plasmids pTα215βen1 and pK2.2, M.Wiles for pB-actin, B.Malissen for $58\alpha^-\beta^-$ cells and G.Sim for providing the GH3 clone. We are very grateful to Jim Kaufman for instruction in biochemical techniques, for valuable discussion of the project, and critical reading of the manuscript. Further experimental support was gratefully received from J.Kirberg, P.Borgulya, W.Swat, U.Chen, H.Karasuyama and K.Karjalainen. The Basel Institute for Immunology was founded and is supported by F.Hoffmann-La Roche & Co. Ltd, CH-4005 Basel, Switzerland.

References

Alarcon,B., Berkhout,B., Breitmeyer,J. and Terhorst,C. (1988) *J. Biol. Chem.*, **263**, 2953–2961.
Ashwell,J.D. and Klausner,R.D. (1990) *Annu. Rev. Immunol.*, **8**, 139–167.
Bonifacino,J.S., Suzuki,C.K., Lippincott-Schwarz,J., Weissman,A.M. and Klaunser,R.D (1989) *J. Cell Biol.*, **109**, 73–83.
Bonifacino,J.S., Cosson,P., Shah,N. and Klausner,R.D (1991) *EMBO J.*, **10**, 2783–2793.
Bonneville,M., Janeway, Jr,C.A., Ito,K., Haser,W., Ishida,I., Nakanishi,N. and Tonegawa,S. (1988) *Nature*, **36**, 479–481.
Chomczynski,P. and Sacchi,N. (1987) *Anal. Biochem.*, **162**, 156–159.
Dennert,G., Hyman,R., Lesley,J. and Trowbridge,I.S. (1980) *Cell. Immunol.*, **53**, 350–364.
Duplay,P., Lancki,D. and Allison,J.P. (1989) *J. Immunol.*, **142**, 2998–3005.
Haars,R., Kronenberg,M., Gallatin,W.M., Weissman,I.L., Owen,F.L. and Hood,L. (1986) *J. Exp. Med.*, **164**, 1–24.
Havran,W.L., Poenie,M., Kimura,J., Tsien,R., Weiss,A. and Allison,J.P. (1987) *Nature*, **300**, 170–173.
Hochstenbach,F. and Brenner,M.B. (1989) *Nature*, **340**, 562–565.
Itohara,S., Nakanishi,N., Kanagawa,O., Kubo,R. and Tonegawa,S. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 5094–5098.
Kincade,P.W., Lee,G., Watanabe,T., Sun,L. and Scheid,M.P. (1981) *J. Immunol.*, **127**, 2262–2268.
Kishi,H., Borguyla,P., Scott,B., Karjalainen,K., Traunecker,A., Kaufman,J. and von Boehmer,H. (1991) *EMBO J.*, **10**, 93–100.
Kitamura,D., Roes,J., Kuehn,R. and Rajewski,K. (1991) *Nature*, **350**, 423–426.
Krimpenfort,P., Ossendorp,F., Borst,J., Melief,C. and Berns,A. (1989) *Nature*, **341**, 742–746.
Kubo,R.T., Born,W., Kappler,J.W., Marrack,P. and Pigeon,M. (1989) *J. Immunol.*, **142**, 2736–2742.
Kudo,A. and Melchers,F. (1987) *EMBO J.*, **6**, 2267–2272.
Lanier,L.L., Yu,G. and Phillips,J.H. (1989) *Nature*, **342**, 803–805.
Leo,O., Foo,M., Sachs,D.H., Samelson,L.E. and Bluestone,J.A. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 1374–1378.
Letourneur,F. and Malissen,B. (1989) *Eur. J. Immunol.*, **9**, 2269–2274.
Lippincott-Schwartz,J., Bonfacino,J.S., Yuan, L.C. and Klausner,R.D. (1988) *Cell* ,**54**, 209–220.
Malek,T.R., Robb,R.J. and Shevach,E.M. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 5694–5698.
Manolios,N., Letourneur,F., Bonifacino,J.S. and Klausner,R.D. (1991) *EMBO J.*, **10**, 1643–1651.
Manz,J., Denis,K., Witte,O., Brinster,R. and Storb,U. (1988) *J. Exp. Med.*, **168**, 1363–1381.
Nussenzweig,M.C., Shaw,A.C., Sinn,E., Danner,D.B., Holmes,K.L. Morse,III,H.C. and Leder,P. (1987) *Science*, **236**, 816–819.
O'Farrell,P.Z., Goodman,H.M. and O'Farrell,P.H. (1977) *Cell*, **1133–1142.**
Palacios,R. and Samaridis,J. (1991) *Mol. Cell. Biol.* in press.
Palacios,R., Studer,S., Samaridis,J. and Pelkonen,J. (1989) *EMBO J.*, **8**, 4053–4063.
Pérez-Aciego,P., Alarcon,B., Arnaiz-Villena,A., Terhorst,C., Timon,M., Seguardo,O.G. and Regueiro,J.R. (1991) *J. Exp. Med.*, **174**, 319–326.
Pillai,S. and Baltimore,D. (1987) *Nature*, **329**, 172–174.
Pillai,S. and Baltimore,D. (1988) *Curr. Top. Microbiol. Immunol.*, **137**, 136–139.
Punt,J.A., Kubo,R.T., Saito,T., Finkel,T.H., Kathiresan,S., Blank,K.J. and Hashimoto,Y. (1991) *J. Exp. Med.*, **174**, 775–783.
Raulet,D.H., Garman,R.D., Saito,H. and Tonegawa,S. (1985) *Nature*, **314**, 103–107.
Reth,M., Petrac,E., Wiese,P., Lobel,L. and Alt,F.W. (1987) *EMBO J.*, **6**, 3299–3305.
Sakaguchi,N. and Melchers,F. (1986) *Nature*, **324**, 579–582.
Schuler,W., Weiler,I.J., Schuler,A., Phillips,R.A. Rosenberg,N., Mak,T.W., Kearney,J.F., Perry,R.P. and Bosma,M.J. (1986) *Cell*, **46**, 963–972.
Sen,J., Arceci,R.J., Jones,W. and Burakoff,S.J. (1989) *Eur. J. Immunol.*, **19**, 1297–1302.
Shores,E.W., Sharrow,S.O., Uppenkamp,I. and Singer,A. (1990) *Eur. J. Immunol.*, **20**, 69–77.
Snodgrass,H.R., Kisielow,P., Kiefer,M., Steinmetz,M. and von Boehmer,H. (1985) *Nature*, **313**, 592–595.
Springer,T.A., Galfrè,G., Secher,D.S. and Milstein,C. (1978) *Eur. J. Immunol.*, **8**, 539–551.
Staerz,U.D., Rammensee,H.-G., Benedetto,J.D. and Bevan,M.J. (1985) *J. Immunol.*, **134**, 3994–4000.
Taylor,A.H., Haberman,A.M., Gerhard,W. and Caton,A.J. (1990) *J. Exp. Med.*, **172**, 1643–1651.
Teh,H.S., Kisielow,P., Scott,B., Kishi,H., Uematsu,Y., Blüthmann,H. and von Boehmer,H. (1988) *Nature*, **35**, 229–233.
Traunecker,A., Dolder,B. and Karjalainen,K. (1986) *Eur. J. Immunol.*, **16**, 851–854.
Trowbridge,I.S., Lesley,J., Schulte,R., Hyman,R. and Trotter,J. (1982) *Immunogenetics*, **15**, 299–312.
Tsubata,T. and Reth,M. (1990) *J. Exp. Med.*, **172**, 973–976.
Uematsu,Y., Ryser,S., Dembić,Z., Borgulya,P., Krimpenfort,P., Berns,A. von Boehmer,H. and Steinmetz,M. (1988) *Cell*, **52**, 831–841.
von Boehmer,H., Bonneville,M., Ishida,I., Ryser,S., Lincoln,G., Smith,R.T., Kishi,H., Scott,B., Kisielow,P. and Tonegawa,S. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 9729–9732.
Wegener,A.K., Letourneur,F., Hoeveler,A., Brocker,T., Luton,F. and Malissen,B. (1992) *Cell*, **68**, 83–95.
Yagüe,J., White,J., Coleclough,C., Kappler,J., Palmer,E. and Marrack,P. (1985) *Cell*, **42**, 81–87.

Received on February 11, 1992; revised on March 20, 1992