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RESEARCH ARTICLE

Analysis and Expression of a Cloned Pre–T Cell Receptor Gene

Claude Saint-Ruf,* Katharina Ungewiss,* Marcus Groettrup, Ludovica Bruno, Hans Joerg Fehling, Harald von Boehmer

The T cell antigen receptor (TCR) β chain regulates early T cell development in the absence of the TCRα chain. The developmentally controlled gene described here encodes the pre-TCRα (pTα) chain, which covalently associates with TCRβ and with the CD3 proteins forms a pre-TCR complex that transduces signals in immature thymocytes. Unlike the λ5 pre–B cell receptor protein, the pTα chain is a type I transmembrane protein whose cytoplasmic tail contains two potential phosphorylation sites and a Src homology 3 (SH3)–domain binding sequence. Pre-TCRα transfection experiments indicated that surface expression of the pre-TCR is controlled by additional developmentally regulated proteins. Identification of the pTα gene represents an essential step in the structure-function analysis of the pre-TCR complex.

T cell development takes place in discrete steps during which the TCR genes are rearranged and expressed in temporal order. During development of TCRαβ-expressing cells the TCRβ gene is rearranged and expressed before the TCRα gene (*1*, *2*). Without TCR rearrangement the development of T cells is arrested at an early stage (*3–5*). By introducing TCRβ transgenes into mice that are defective for rearrangement of antigen receptor genes, it was shown that TCRβ proteins, in the absence of TCRα chains, are sufficient to promote early T cell development (*6–8*). Although such mice are still rearrangement-defective, their immature thymocytes (which express neither the CD4 nor CD8 proteins) begin to express CD4 and CD8 coreceptors, transcripts of the TCRα locus become detectable (*7*), and the number of thymocytes increases (*6–8*). Introduction of TCRβ transgenes into normal mice suppresses rearrangement of endogenous TCRβ genes (*9*, *10*). The TCRβ transgene is expressed on the cell surface in the absence of TCRα proteins in both normal (*11*) as well as in rearrangement-defective mice (*7*, *8*, *12*) in an 80-kD disulfied-linked complex and as a glycosyl-phosphatidylinositol (GPI)–linked 40-kD monomer.

The presence of the TCRβ chain in the 80-kD complex suggested that either the complex was a homodimer or that an unknown TCR chain was involved that may affect T cell maturation. A glycosylated chain of 33 kD (gp33) is paired with TCRβ proteins in a TCRβ-transfected immature T cell line (SCB.29) from severe combined immunodeficient (SCID) mice (*12*), but could not be identified in normal thymocytes (*12*, *13*). The gp33-TCRβ complex of SCB.29 cells is associated with CD3 proteins (*8*, *12*) and cross-linking of TCR chains initiates Ca^{2+} mobilization. This suggested that this TCRβ complex could be responsible for the developmental progression observed in TCRβ transgenic, rearrangement-deficient mice, whereas the TCRβ GPI-linked monomer could represent a transgenic artifact (*14*, *15*). We have now cloned the gene encoding gp33 and examined its structure and expression. Because of its properties, the gp33 protein was named the pre-TCRα (pTα) chain.

Pre–T cell receptor α (pTα) expression in immature T cells. The pTα chain can be identified by two-dimensional (diagonal) gel electrophoresis, in which the disulfide-linked pTα protein under reducing conditions migrates away from the diagonal just underneath the TCRβ protein (*12*) (Figs. 1 and 2). The analytical method was scaled up to obtain sufficient amounts of pTα protein for microsequencing. In a first attempt a 20-amino-acid-long NH_2-terminal sequence was obtained; a peptide of the 18 NH_2-terminal residues was synthesized and injected into rabbits to obtain a pTα-specific antiserum. The antiserum was tested for binding to the pTα protein. To this end lysates from the TCRα-negative SCB.29 cell line as well as the TCRαβ-expressing B6.2.16BW hybridoma (*12*) were precipitated with the monoclonal antibody (mAb) F23.1 to $V_\beta 8$ proteins (*16*). Precipitates

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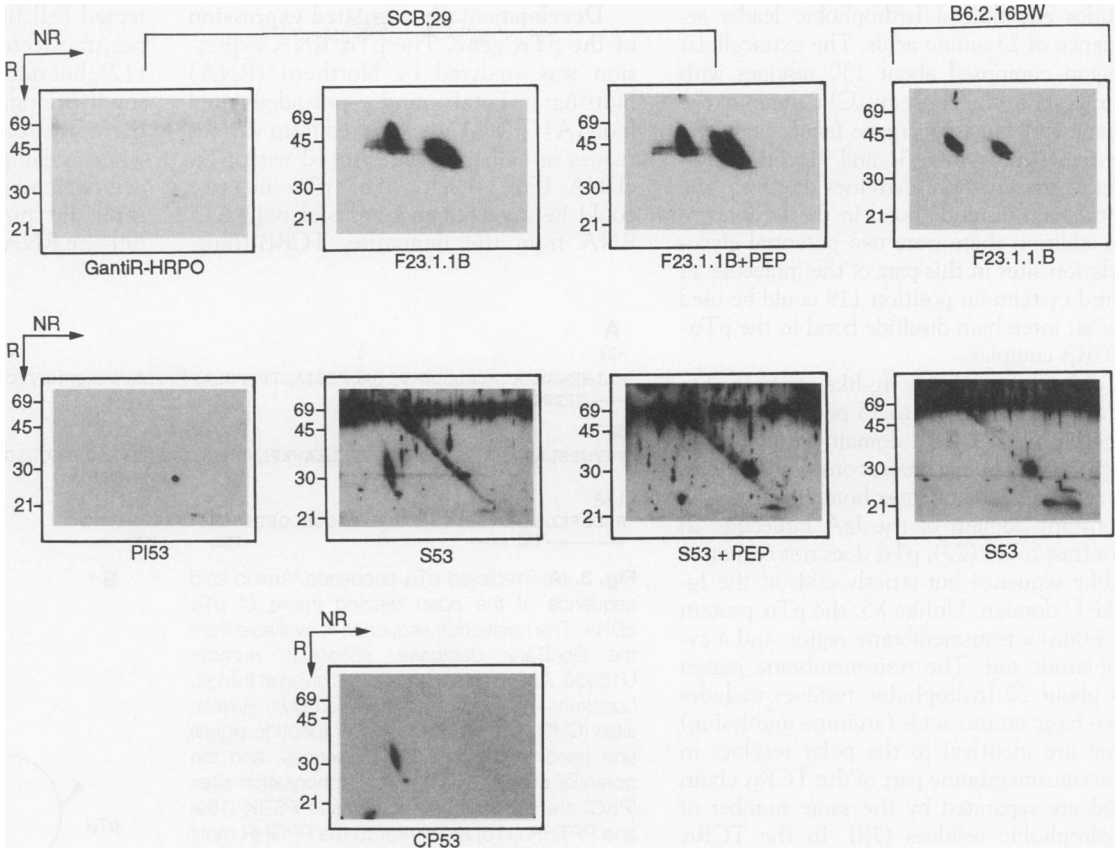

Fig. 1. Identification of the pTα chain by antibodies. Immunoprecipitations of TCRβ from either SCB.29 or B6.2.16BW cells were immunoblotted with control reagents or antibodies to TCRβ or pTα, with or without peptide (PEP) from the NH_2-terminus of pTα (*38*). SCB.29, cell line from SCID mouse transfected with TCRβ; B6.2.16BW, TCRαβ-expressing T cell hybridoma; GantiR-HRPO, goat antibodies to rabbit immunoglobulin conjugated to horseradish peroxidase; F23.1, mAb to TCRβ ($V_\beta 8$); PI53, pre-immune serum from rabbit 53; S53, immune serum from rabbit 53 injected with NH_2-terminal peptide of pTα; CP53, affinity column–purified S53. Molecular sizes are given in kilodaltons.

were separated on diagonal gels and the proteins analyzed in Western immunoblots by staining with either mAb F23.1 or serum from immunized rabbit 53 (Fig. 1). The TCRβ mAb stained proteins off and on the diagonal, corresponding to disulfide-linked and -unlinked TCRβ proteins, respectively, in the lysates of both SCB.29 and B6.2.16.BW cells (Fig. 1, top) whereas the control GantiR-HRPO [goat anti-rabbit immunoglobulin (Ig) conjugated to horseradish peroxidase] produced no signal. The pre-immune serum from rabbit 53 (PI53) likewise produced no signal (except for some weak reactivity with the 30-kD marker on the diagonal) whereas the immune serum (S53) stained in SCB.29 lysates a protein off the diagonal beneath the TCRβ protein that was not stained in B6.2.16BW lysates (Fig. 1, middle). The staining of the pTα protein could be completely inhibited by the specific peptide, whereas the extensive background staining could not. Affinity purification on a peptide column finally yielded a reagent of exquisite specificity for the pTα protein that became visible as a streak of differentially glycosylated proteins as well as a nonglycosylated dot (Fig. 1, bottom). These results established that the protein sequence was obtained from the pTα protein and that the antiserum could be used to analyze expression of the pTα-TCRβ complex in various cells.

Because the pTα-TCRβ complex in thymocytes was not previously identified, we used the above approach to examine this complex in thymocytes from TCRα-deficient ($TCR\alpha^{-/-}$) mice (Fig. 2). We could detect the pTα-TCRβ complex by precipitation with the mAb H57 to all TCRβ proteins (*17*) from lysates of $TCR\alpha^{-/-}$ thymocytes. Thus the pTα-TCRβ complex does not represent a peculiarity of a single cell line, but is also formed and glycosylated in immature thymocytes.

Cloning of the pTα chain complementary DNA (cDNA). To obtain a pTα chain cDNA sequence we prepared a cDNA library from the SCB.29 cell line in lambda ZAP II (Stratagene). A lysate of the library was prepared and amplified by PCR (polymerase chain reaction) with degenerate or nondegenerate oligonucleotides, corresponding to the most 3′ part of the pTα NH_2-terminal protein sequence and an oligonucleotide complementary to the lambda ZAP vector-sequence at the 5′ end of the cDNA inserts. Specific PCR products were identified by Southern (DNA) hybridization with another set of degenerate oligonucleotides corresponding to the pTα sequence between the PCR primers. Hybridizing PCR fragments were subcloned and sequenced. In this way a fragment of approximately 180 bp was obtained that encoded part of the 5′ untranslated region, the leader sequence, and the sequence corresponding to the first five amino acids of the pTα protein. This fragment was then used as a probe for conventional screening of the SCB.29 cDNA library. Several independent lambda clones were isolated that contained the full-length coding sequence of pTα. The sequence revealed an open reading frame of 618 nucle-

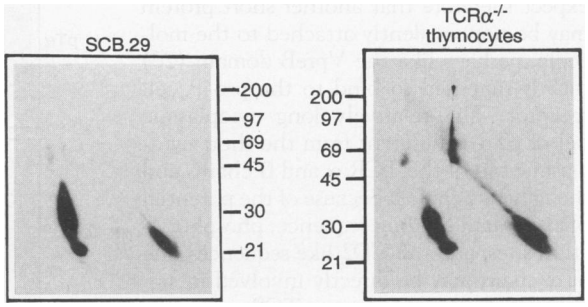

Fig. 2. Identification of the pTα chain in $TCR\alpha^{-/-}$ thymocytes (4×10^9 cells) and SCB.29 (2×10^7 cells). Two-dimensional SDS-PAGE of immunoprecipitates with H57 (*17*). The pTα chain is revealed by CP53 serum. The spot on the diagonal may represent nondisulfide-linked pTα in the precipitate. Staining with mAb F23.1 was similar to that in Fig. 1. Methods were as in Fig. 1. Molecular sizes are indicated in kilodaltons.

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otides encoding a hydrophobic leader sequence of 23 amino acids. The extracellular region comprised about 130 residues with similarity to the constant (C) domain of the immunoglobulin supergene family with two cysteines in position 31 and 91 corresponding to the invariant cysteines that form the intrachain disulfide bond in the Ig domain. In addition there were two potential glycosylation sites in this part of the molecule. A third cysteine in position 119 could be used for an interchain disulfide bond in the pTα-TCRβ complex.

The extracellular Ig-like domain has only some weak (20 to 25 percent) homology with the Ig-like domain of TCRα, Ig light and Ig heavy chain constant domains, as well as λ5, being most homologous to the constant domain of the IgA molecule. In contrast to λ5 (*20*), pTα does not contain a J-like sequence but strictly ends at the Ig-like C domain. Unlike λ5, the pTα protein contains a transmembrane region and a cytoplasmic tail. The transmembrane region of about 20 hydrophobic residues includes two basic amino acids (arginine and lysine) that are identical to the polar residues in the transmembrane part of the TCRα chain and are separated by the same number of hydrophobic residues (*18*). In the TCRα chain, these polar residues are essential for the assembly and transport of the TCRαβ-CD3 complex (*18*). The cytoplasmic region of about 31 residues is rich in proline residues and could constitute a SH3-domain binding region. In addition, there are PPSRK and PPTHR sequences similar to the PPGHR motif present in the cytoplasmic tail of CD2, known to be involved in CD2-dependent T cell activation (*19*) and containing two potential phosphorylation sites for protein kinase C (Fig. 3).

The sequence analysis indicates that the pTα protein is well suited for pairing with the TCRβ chain. Even though there is only moderate identity to any particular member of the Ig supergene family, this protein bears the hallmarks of this family. The cysteine in position 119 as well as the transmembrane part seem designed to form a complex with TCRβ and CD3 proteins. The pTα-TCRβ heterodimer has an asymmetrical shape because the TCRβ has two and the pTα only one extracellular Ig-like domain. One might expect therefore that another short protein may be noncovalently attached to the molecule, perhaps like the VpreB domain (*20*) that is supposed to bind to the pre–B cell receptor. The relatively long cytoplasmic tail of pTα is different from the short cytoplasmic tail of the TCR α and β chains and the Ig heavy chains. Because of the potential SH3-domain binding sequence, phosphorylation sites, and the CD2 like sequences, the pTα chain may be directly involved in signal transduction by the pre-TCR.

Developmentally regulated expression of the pTα gene. The pTα RNA expression was analyzed by Northern (RNA) blotting: Total and polyadenylated [poly(A)$^+$] RNA was isolated from various tissues or cell lines and probed with pTα cDNA (Fig. 4, left). The pTα message could be detected in total and poly(A)$^+$ RNA from the immature, TCRβ-transfected cell line SCB.29 as well as in the nontransfected parental line SCI.ET.27F (*12*), but not at all in the TCRαβ-expressing hybridoma B6.2.16.BW (*12*). In mice, there was some expression in the thymus, whereas lymph node (Fig. 4) and spleen cells were negative. Expression of the pTα gene did not require rearrangement, as full-size RNA was abundant in the thymus

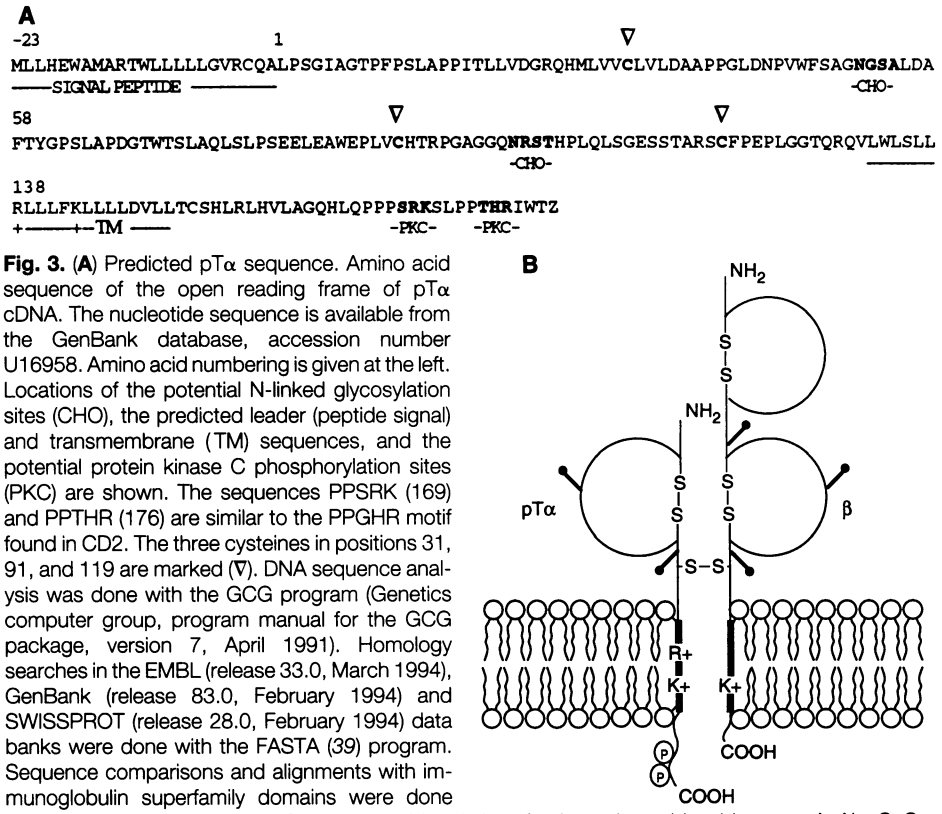

Fig. 3. (**A**) Predicted pTα sequence. Amino acid sequence of the open reading frame of pTα cDNA. The nucleotide sequence is available from the GenBank database, accession number U16958. Amino acid numbering is given at the left. Locations of the potential N-linked glycosylation sites (CHO), the predicted leader (peptide signal) and transmembrane (TM) sequences, and the potential protein kinase C phosphorylation sites (PKC) are shown. The sequences PPSRK (169) and PPTHR (176) are similar to the PPGHR motif found in CD2. The three cysteines in positions 31, 91, and 119 are marked (∇). DNA sequence analysis was done with the GCG program (Genetics computer group, program manual for the GCG package, version 7, April 1991). Homology searches in the EMBL (release 33.0, March 1994), GenBank (release 83.0, February 1994) and SWISSPROT (release 28.0, February 1994) data banks were done with the FASTA (*39*) program. Sequence comparisons and alignments with immunoglobulin superfamily domains were done with the PILEUP (*40*) program. Single-letter abbreviations for the amino acid residues are: 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; and Y, Tyr. (**B**) The pTα-TCRβ complex. ▬, Corresponds to the N-linked glycosylation sites. P, represents protein kinase C phosphorylation sites in the pTα cytoplasmic tail. Two basic amino acids, arginine (R$^+$) and lysine (K$^+$), are located in the pTα transmembrane region.

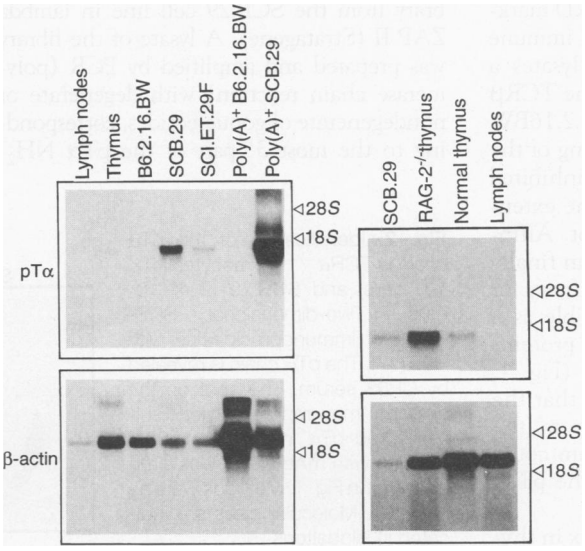

Fig. 4. pTα mRNA expression in various cells and tissues. Total or poly(A)$^+$ RNA was isolated from cell lines and tissues and the RNA was separated on agarose gels. After transfer, the RNA was probed with a labeled cDNA specific for pTα; after the original probe was stripped from the filter, it was again hybridized with a labeled β-actin probe (*41*).

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of RAG-2$^{-/-}$ (recombination activating gene 2) rearrangement-defective mice (*21*) and it was expressed higher in RAG-2$^{-/-}$ thymocytes than in thymocytes from normal mice (Fig. 4). The latter result suggested that the pTα gene was predominantly expressed in the more immature thymocytes.

Expression in early thymocytes was confirmed by PCR analysis with oligonucleotides specific for the pTα gene (Fig. 5). The developmentally regulated pTα message was expressed in different amounts at successive stages of thymic development. There was high expression in CD44$^-$CD25$^+$ and CD44$^-$CD25$^-$ double negative (CD4$^-$CD8$^-$) cells but there was weaker expression in earlier and later stages of development. Small single positive (expressing only CD4 or CD8) thymocytes had no detectable pTα RNA. The difference in the intensity of the pTα RNA bands between RAG-2$^{-/-}$ and normal thymocytes in Fig. 4 is explained by the fact that most cells in the normal thymus are small CD4$^+$CD8$^+$ cells, whereas in the RAG-2$^{-/-}$ thymus most cells are CD44$^-$CD25$^+$ and because CD44$^-$ CD25$^+$ cells express more pTα RNA than small CD4$^+$CD8$^+$ thymocytes. Thus pTα is expressed in the thymus before TCRβ and TCRα genes, but there may be some overlap in the expression of pTα and TCRα genes. Coexpression of pTα-TCRβ and TCRα-TCRβ heterodimers on the cell surface of TCRα-transfected SCB.29 cells has been observed (*22*). Therefore this coexpression is theoretically possible in immature thymocytes.

The transfection of pTα in a TCRα-deficient, mature T cell line. The above results show that a glycosylated pTα-TCRβ complex is transported to the cell surface in immature T cells. Because TCRβ has two and pTα only one extracellular Ig-like domain, one would expect that the pTα-TCRβ complex has an additional, noncovalently associated Ig-like domain, much like the VpreB domain in the pre–B cell receptor (*23*). One might further expect that without such an additional protein the pTα-TCRβ complex may not be transported to the cell surface.

To test whether or not the pTα-TCRβ complex was sufficient for cell surface expression, we transfected a pTα construct into the 58α$^-$βy.1 mature T cell line (*8*), which expresses a TCRβ gene and CD3 proteins that are essential for surface expression of the αβ TCR, but lacks TCRα chains. When these cells were supertransfected with the pTα construct, intracellular pTα-TCRβ dimers were observed, but in contrast to dimers in the immature T cell line SCB.29, the disulfide-linked pTα proteins in the mature T cell line were poorly or not at all glycosylated, indicating that most of the heterodimer did not leave the endoplasmic reticulum (Fig. 6). Also, surface expression of TCRβ in the pTα transfectants was not increased, whereas TCRαβ surface expression was observed after TCRα transfection. This result was consistent with the hypothesis that another developmentally regulated protein (VpreT) may be required for proper assembly, glycosylation, and transport to the cell surface of the pTα-TCRβ complex. The result is of course equally consistent with the hypothesis that mature T cells may contain proteins that prevent pTα-TCRβ complexes from leaving the endoplasmic reticulum and that the pTα-TCRβ complex can only be transported to the cell surface in the absence of these retention molecules.

Regulation of T cell development by the pre-TCR complex. In conjunction with experiments in TCRβ transgenic, rearrangement-defective SCID mice (*6–8*) and analogous experiments in TCRβ transgenic RAG$^{-/-}$ mice (*21, 24*), our results are consistent with the hypothesis that the pre-TCR complex is sufficient to promote T cell development in the absence of other TCR chains encoded by rearranging genes. Although the experiments in the TCRβ transgenic, rearrangement-deficient mice have indicated that TCRβ proteins are sufficient to induce T cell maturation, studies in TCRβ$^{-/-}$ mice suggest that a TCRβ protein is not essential for the expression of CD4 and CD8 molecules in development because CD4$^+$CD8$^+$ cells are present in TCRβ$^{-/-}$ mice (*24*). The possibility was considered that CD4$^+$CD8$^+$ thymocytes in TCRβ$^{-/-}$ mice were of the γδ lineage (because they were absent in TCRβ$^{-/-}$ TCRγ$^{-/-}$ mice) and that the TCRβ protein was essential for expression of CD4 or CD8 coreceptors in the αβ lineage (*24*). There are, however, no experiments supporting this view. In contrast, reconstitution of thymuses from SCID (*25, 26*) or RAG$^{-/-}$ mice (*22*) with either pro-T cells or γδT cells have shown that thymocytes devoid of TCR proteins can express CD4 and CD8 molecules as long as other TCR-bearing cells are present. Thus the pre-TCR complex may not directly regulate CD4 and CD8 expression through intracellular signaling.

Intracellular signaling by the pre-TCR complex may, however, be essential to obtain large numbers of immature thymocytes. This can be deduced from experiments that showed that the proportion of productive

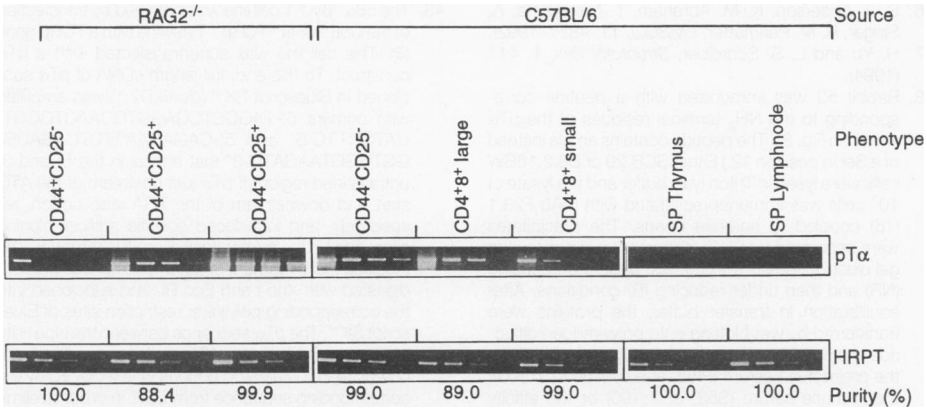

Fig. 5. Semiquantitative analysis of pTα expression on lymphocyte subsets. Thymocytes from RAG-2$^{-/-}$ and normal C57BL/6 mice were separated by fluorescence-activated cell sorting (FACS) or complement-dependent killing. Subsets of T cells were subjected to polymer chain reaction (PCR) as described in (*42*). Samples were stained with ethidium bromide. SP, single positive (CD4 or CD8) T cells; HPRT, hypoxanthine-guanine phosphoribosyl transferase.

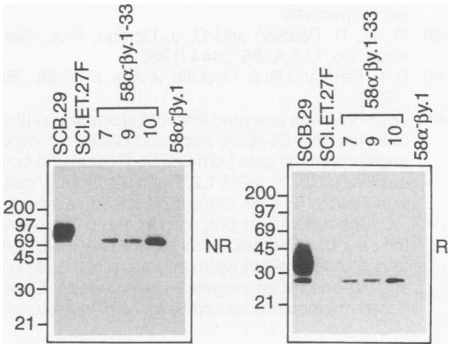

Fig. 6. Transfection of a pTα construct into a mature, TCRα-deficient T cell line. Immunoprecipitations of TCRβ from SCB.29, SCI.ET.27F (the nontransfected SCID cell line), the pTα transfectants of 58α$^-$βy.1 (a TCRα-mature T cell line) (*43*) named 58α$^-$βy.1-33 and represented by clones 7, 9, and 10, and 58α$^-$βy.1 cells were separated under nonreducing (NR) and reducing (R) conditions and immunoblotted with the CP33 pTα antiserum.

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rearrangements in $CD44^-CD25^-$ (*28*) and total thymocytes (*29*) from $TCR\alpha^{-/-}$ mice were much higher than expected if cells of this phenotype could similarly expand, irrespective of whether or not they carried productive TCRβ genes. Thus the pre-TCR complex may regulate survival and expansion of immature T cells rather than regulating directly CD4 and CD8 gene expression in the lineage of αβ T cells.

The role of the pre-TCR complex in T cell development is supported by other studies. In SCID (*30*) as well as in TCRβ- or RAG-deficient (*31*, *32*) mice, the effects of productive TCRβ transgenes on thymocyte development can be mimicked by treating thymocytes of these mice with antibodies to CD3ε. These results support the hypothesis that the TCRβ protein exerts its function through a pre-TCR complex that associates with CD3 proteins. It is not clear from these studies whether signaling through CD3 is all that is required or whether the cytoplasmic part of the pTα chain plays an essential role. A potential role of the cytoplasmic tail of pTα needs to be analyzed under more physiological conditions.

A role of $p56^{lck}$ in the signaling by the pre-TCR is implicated by studies in mice that are Lck-defective (*33*) or carry a dominant negative Lck mutation (*34*). The Lck dominant negative mutation results in a developmental arrest that resembles that of rearrangement-deficient mice. On the other hand the introduction of an active *lck* gene in rearrangement-deficient mice (*35*) or in normal mice (*36*) causes the same effects as a TCRβ transgene. Thus $p56^{lck}$ may be part of the signaling cascade of the pre-TCR complex. Because the cytoplasmic tail of the pTα chain contains a proline-rich, potential SH3-binding domain (*37*), $p56^{lck}$ may be recruited by the pTα protein into the pre-TCR complex.

The cloning and expression analysis of the pTα gene has established that the pre-TCR is expressed in immature thymocytes and opens the way to determine the precise structure and function of the pre-TCR complex in T cell development.

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38. Rabbit 53 was immunized with a peptide corresponding to the NH_2-terminal residues of the pTα shown in Fig. 3. (The peptide contains an Ala instead of a Ser in position 12.) Either SCB.29 or B6.2.16BW cells were lysed in Triton lysis buffer and the lysate of 10^7 cells was immunoprecipitated with mAb F23.1 (*16*) coupled to agarose beads. The precipitates were separated by SDS–10 percent polyacrylamide gel electrophoresis (PAGE), first under nonreducing (NR) and then under reducing (R) conditions. After equilibration in transfer buffer, the proteins were transferred by wet blotting onto polyvinyliden difluoride membranes. Filters were incubated with either the preimmune serum (PI53, at a dilution of 1:100), the immune serum (S53, at 1:100) or the affinity column–purified (CP53, at 1:100) in phosphate-buffered saline containing 0.4 percent Tween and 2 percent fetal calf serum. For peptide competition, peptide (35 μg/ml) was first incubated with S53 serum or mAb F23.1 for 1 hour. For detection of TCRβ protein a F23.1-biotin conjugate was used. After the first-stage reagents were removed, GantiR-HRPO at 1:20,000 or streptavidine-HRPO (1:5000) was added for visualization of the rabbit and F23.1 antibodies, respectively.
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41. Total RNA was prepared from cell suspensions (thymocytes from C57BL/6 mice and $RAG\text{-}2^{-/-}$ mice, and lymph node cells from C57BL/6 mice) and from cell lines (SCB.29, SCI.ET.27F and B6.216.BW cells) with RNAzol B (TM Cinna Scientific, Friedswood, TX). The mRNA was prepared from total RNA with the use of the Oligotex TM mRNA Kit (QIAGEN). Both total RNA and mRNA were separated on 1.2 percent agarose–formaldehyde gels and transferred to GeneScreen membranes according to the manufacturer's recommendations (Du Pont). Filters were first hybridized at 65°C for 8 hours and hybridized with the same solution [1 mM EDTA, 0.5 M NaH_2PO_4, 7 percent SDS, and salmon sperm DNA (100 μg/ml)] containing the specific probe at 65°C overnight. To remove any unspecifically bound probe, the membranes were washed first with 1 mM EDTA, 40 mM NaH_2PO_4, 1 percent SDS at 65°C. Filters were hybridized first with a cDNA probe corresponding to the entire coding sequence of pTα and subsequently, after dishybridization, with a probe specific for β-actin message. Washed membranes were exposed to Kodak X-OMAT AR film at −70°C for 1 to 5 days.
42. $CD44^+CD25^-$, $CD44^+CD25^+$, and $CD44^-CD25^+$ double-negative ($CD4^-CD8^-$) thymocytes were obtained from $RAG\text{-}2^{-/-}$ mice. $CD44^-CD25^-$ thymocytes were obtained from C57BL/6 mice by complement-dependent killing of thymocytes with antibodies to CD4 and CD8. The thymocytes were then stained with antibodies specific for CD4, CD8, CD44, CD25, and CD3 surface markers to obtain the desired populations by cell sorting. The purity of the subsets was deduced from reanalysis of sorted cells. In order to quantitate pTα RNA, cells (5×10^4) were directly sorted in 500 μl of RNAzol (TM Cinna Scientific), and total RNA was extracted according to the manufacturer's protocol. The cDNA was prepared with random hexamer primers and reverse-transcribed with Superscript kit (Gibco BRL). Dilutions (1:3) of cDNA in water were then used in PCR amplification reactions. Primers used were oligonucleotides recognizing sequences in the 5′ and 3′ regions of the pTα (5′-CTGCAACTGGGTCATGCTTC-3′ and 5′ TCAGACGGGTGGGTAAGATC-3′) and HRPT (5′-CACAGGACTAGAACACCTGC-3′ and 5′-GCTGGTGAAAAGGACCTCT-3′) genes. Amplification was done for 35 cycles at an annealing temperature of 55°C with a thermal cycling machine (Perkin-Elmer Cetus). A 15-μl portion of each amplified product was separated through a 1.2% agarose gel by electrophoresis and stained with ethidium bromide.
43. The $58\alpha^-\beta y.1$ T cell line was obtained by transfection of the C58 $TCR\alpha^-TCR\beta^-$ T cell line with a TCRβ gene (*8*). This cell line was supertransfected with a pTα construct. To this end, full-length cDNA of pTα subcloned in Bluescript SK^+ (clone D2.1) was amplified with primers 5′-TAGCCTCGAGCTGCAACTGGGTCATGCTTC-3′ and 5′-CAGAGAATTCTCAGACGGGTGGGTAAGATC-3′ that anneal in the 5′ and 3′ untranslated region of pTα just upstream of the ATG start and downstream of the TGA stop codon, respectively, and introduced specific artificial cloning sites: Xho I in the former case, Eco RI in the latter. The resulting PCR fragment of about 660 bp was purified, digested with Xho I and Eco RI, and subcloned into the corresponding polylinker restriction sites of Bluescript SK^+. The pTα sequence between the Nco I site at the second in-frame ATG and the one Bgl II site downstream of the stop codons was replaced by the corresponding sequence from D2.1 in order to eliminate potential mutations introduced during the PCR amplification. The resulting plasmid was digested with Xho I and Not I to release the cDNA fragment encoding pTα, which was then inserted in the corresponding sites of expression vector BCMGSHyg (*20*). The construct was transfected into $58\alpha^-\beta y.1$ cells by electroporation.

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