Genomic structure of the human pre-T cell receptor alpha chain and expression of two mRNA isoforms

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The pre-TCR, which is minimally composed of the TCRβ chain, the pre-Tα chain, and the CD3 complex, regulates early T cell development. The pre-Tα chain is a 33-kDa type I transmembrane glycoprotein with an extracellular part similar to the constant domain of the immunoglobulin supergene family. We have sequenced (11 kb) the human pTα gene, which like the murine pTα gene consists of four exons: exon 1 encodes the 5' untranslated region, the leader peptide and the first three amino acids of the mature protein, exon 2 the extracellular immunoglobulin (Ig)-like domain, exon 3 a 15-amino acid peptide including a cysteine required for heterodimerization with TCRβ, exon 4 the transmembrane region, the cytoplasmic tail and the 3' untranslated sequence. The human pTα gene is located on chromosome 6p21.3, close to the HLA-A locus. Reverse transcription-PCR studies with human thymus and leukemic cells showed that alternative splicing produces a shorter pTα isoform, which lacks the Ig-like domain but contains the transmembrane elements and the extracytoplasmic cystein and which could thus permit pairing with TCRβ chain and association with CD3 molecules. The conserved splice sites suggest a yet ill-defined biological function of the short pTα protein.

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1 Introduction

In the thymus T cells develop from precursor cells in discrete steps that are controlled by the pre-TCR as well as the αβ TCR for antigen. The pre-TCR consists minimally of the TCRβ chain and the covalently bound pre-TCRα chain associated with signal-transducing proteins of the CD3 complex [1]. The pre-TCR rescues developing T cells that harbor a productive TCRβ rearrangement from programmed cell death and induces extensive cell division as well as differentiation. It is also essential for allelic exclusion of the TCRβ locus through feedback inhibition of TCRβ rearrangement (reviewed in [2]). Finally, it appears to play an important role in directing T cell development into the αβ pathway [3].

Both murine [4] and human [5] pTα cDNA have been cloned. The deduced amino acid (aa) sequences revealed a type I transmembrane protein consisting of a hydrophobic signal peptide, an extracellular domain with a structure similar to the constant (C) domain of the immunoglobulin (Ig) supergene family, hydrophobic transmembrane region, and a cytoplasmic tail. In contrast to the short (3 aa) cytoplasmic domains of the TCRα and TCRβ chain, pTα has a prominent cytoplasmic tail (30 aa in mice and 114 aa in humans). Additionally, the pTα is not subjected to the rearrangement process and it is devoid of J-like sequences as well as V-like Ig domains [4, 6]. With the exception of the cytoplasmic domain, murine and human pTα cDNA are very homologous (70–80 %). Murine pTα is located in the D/E1 region on chromosome 17. The human gene resides in the syntenic region p21.2-p12 on chromosome 6 close to the MHC.

In the present study we present data on the human pTα gene cloned from a human chromosome 6 cosmid library. To define some conserved regions potentially important for gene regulation the complete human pTα gene sequence was established and compared with the murine sequence. Analysis of pTα expression in human thymus and in leukemic human cell lines shows that a splice variant pTα isoform is expressed. This isoform, which lacks the Ig-like domain could also be found in mice and therefore may have an important function in T cell development.

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2 Results and discussion

2.1 Isolation of genomic clones encoding the pTα gene

A cosmid library of human chromosome 6 DNA was purchased from the Reference Library Database (RFLB) of the Max Planck Institute for Molecular Genetics (Berlin, Germany). Nizetic et al. [7] constructed the library from DNA digests of flow-sorted human chromosomes 6 of the 4× cell line which were ligated into lawris 4. DH5 alpha MCR (BRL) was used as host. Subsequently, a high-density colony filter (~ 36 864 clones) corresponding to this library, was hybridized with a human pTα cDNA probe. Ten clones were found to be pTα positive. One of them co-hybridizes with a probe designated Alu yac 7 (RFLD data), which is ALU PCR-derived genomic. This clone is HLA-A positive and located on 6p21-3 [8], thereby indicating the localization of the pTα gene. Previous *in situ* hybridization data already mapped it to the 6p21-6p12 region of the human genome [5].

Positive clones for pTα were mapped by restriction digestion and Southern blotting. Three of them, containing the complete pTα gene, were extensively studied. No difference was found regarding the organization of pTα in these three recombinants. A pTα restriction map is shown in Fig. 1. For two directional sequencing we subcloned inserts of 34 kb, 44 kb, and 46 kb, respectively into bluescript KS$^+$ plasmids.

2.2 Sequence and organization of the human pTα gene

Different subclones were sequenced using the "abiprism" system (PE Applied Biosystems, GB). We sequenced 11 kb starting 1500 bp upstream the ATG codon and ending 250 bp downstream the stop codon (sequence is available under accession number AF084941). Comparing this genomic sequence with pTα cDNA revealed the following exon/intron organization (Fig. 2).

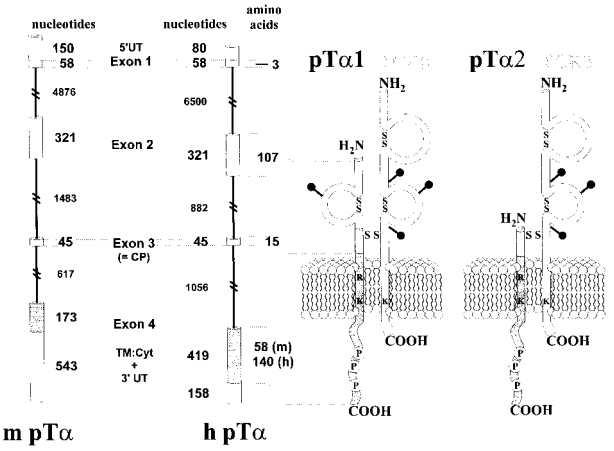

Figure 2. Genomic and protein organization of murine and human pTα

Human pTα is encoded by four exons, which correspond, as in most other Ig superfamily genes, approximately to the different domains of the protein. The same organization has been observed for murine pTα. The first exon contains the 5' untranslated sequence, the leader peptide and the first three aa of the mature protein. In humans the following intron 1 is 6.5 kb long (5 kb in mice). It is particularly rich in repetitive DNA: 16 SINE elements (16 ALU sequences) and 3 LINE elements (2 LINE-2 and 1 LINE-1). Five simple repeats and 2 low complexity sequences have also been noticed. Exon 2 encodes 105 aa of the extracellular Ig-like domain, including the two cysteines that are thought to form the typical Ig intrachain disulfide bridge. It is followed by intron 2 (887 bp) containing one SINE element (mir) Exon 3 encodes 16 aa, which form the connecting peptide con-

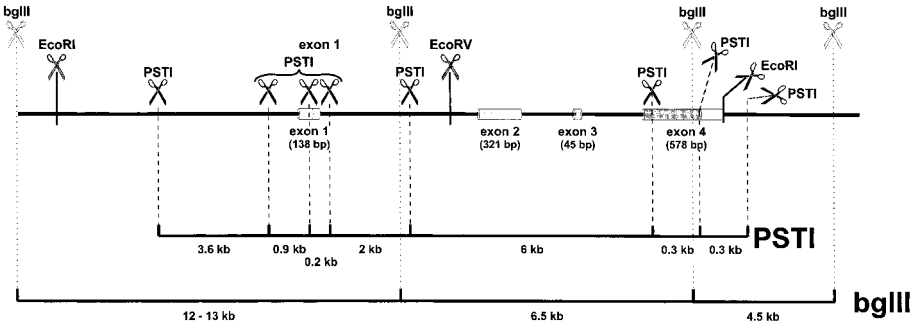

Figure 1. Restriction map of the human pTα gene

taining the cysteine residues for dimerization with the TCRβ chain. Exon 2 and exon 3 have the same size in humans and mice. The following intron contains 1057 bp encompassing a SINE element (Alu Sg). Exon 4 encodes 139 aa, and contains the sequence for the approximately 20-aa long transmembrane region and the 3' untranslated region of ~ 160 nucleotides. This includes two positively charged aa (arginine, lysine) which are located at the same position in the murine pTα chain but also in the TCRα and TCRδ chain. These arginine and lysine are thought to be essential for the interaction of the TCR chains with negatively charged residues in the transmembrane regions of the CD3 complex [9]. The last 114 aa form the cytoplasmic tail. While more than 80 % aa identity can be observed between the murine and human Ig-like and transmembrane region, no significant homology could be found between the 114-aa human cytoplasmic tail and the 30 residues of the murine pTα cytoplasmic tail. We have suggested [5, 6] that the lack of identity between the human and murine cytoplasmic tails could reflect a divergence in gene structure. In fact, the organization of the pTα genome is very similar to the constant regions of TCRα and TCRδ genes. However, two differences can be found. First, only pTα is endowed with a leader sequence located far from the next coding sequence. This could be explained by the fact that pTα is expressed without a second, V region-like Ig domain. Second, the pTα transmembrane region, the cytoplasmic tail, and the 3' untranslated region are encoded by a single exon. In TCRα and TCRδ genes the 3' untranslated region is encoded by a separate exon. In mice the 3' untranslated region contains a B2 repetitive element which provides the polyadenylation site and thus can be considered as an integral part of the pTα gene. We have suggested that the specific structure of the murine pTα gene could be due to the presence of this transposon-like element in the 3' end of the gene [6]. Because human pTα has the same organization but has lost the B2 repetitive elements, this assumption is no longer valid.

2.3 Regulatory elements of the pTα gene

We sequenced (1.5 kb) upstream of the ATG start codon of the pTα gene. Usually promoter sequences are present within 100 bp upstream of the ATG start codon. While this region is well conserved between humans and mice (70 % of nucleotide identity) no TATA or CCAAT like regulatory elements, which are typical promoter motifs of many non-housekeeping genes, could be found. Furthermore, computer-based sequence analysis did not permit to define a potential promoter. Further analyses of this region by deletion and/or mutagenesis experiments will be necessary to define the promoter site and further regulatory elements.

Intron 1 is very large compared to other Ig-family genes (6.5 kb for the human and 5 kb for the murine gene). In the middle of this intron a single, 80-bp long sequence is highly conserved between humans and mice (80 % homology). Since intronic sequences are usually not conserved between species, except if they contain some regulatory elements, this sequence could play a role in the regulation of pTα. A detailed analysis using the TESS program (BCM Search Launcher) revealed potential binding sites for transcription factors AP1 and μ-EBP-C2. AP1 family members are known to play a critical role in regulating T cell gene expression in combination with other transcription factors [10]. Binding sites for μ-EBP-C2 have been found in intragenic enhancers of TCRβ and IgH [11, 12]. Like in mice, the human pTα intron 1 is heavily loaded with short interspersed repetitive elements. About 4.5 kb of the 6.6 kb of human intron 1 are repeat sequences. The conservation of these sequences among humans and mice additionally suggests a role of intron 1 in the regulation of pTα expression. Since LINE elements carry an internal split promoter for RNA polymerase III, they are actively transcribed by this polymerase. They participate in host gene activity regulation by inserting into regulatory elements or by providing regulatory elements themselves [13, 14]. Therefore, it would be interesting to analyze whether this region plays an important role in the regulation of pTα expression. This could be helpful to understand the commitment of T cells to either the αβ or the γδ lineage since it appears that γδ precursors but not γδ T cells express the pTα gene [3].

2.4 Expression of human pTα and evidence for a splice-variant

We have used RT-PCR to analyze expression of human pTα in human thymus and in the human HPB-ALL, MOLT-4 and Jurkat cell line. Primers have been chosen in exon 1 and exon 4 in order to amplify the entire cDNA. Specific transcripts were found in the human thymus, in HPB-ALL and MOLT-4 cells, respectively. The Jurkat cell line was pTα negative. In all positive samples we observed two different bands (Fig. 3). One of the expected size of ~ 640 bp, and a smaller one of ~ 320 bp. Sequencing of these bands revealed that the larger one corresponds to the originally described form of pTα (as described in [5]), designated pTα1. The smaller band represents a splice-variant of pTα, designated pTα2.

The splice variant sequence, pTα2, shows an open reading frame. The deduced primary sequence contains 231 aa. Comparison with pTα1 revealed that pTα2 lacks the major part of the Ig-like domain. During pTα2 splicing, which uses at the same splice sites as pTα1, the

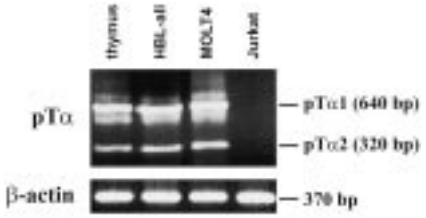

Figure 3. Expression of two different pTα splice variants. pTα expression was analyzed by RT-PCR in human thymus and three different human acute lymphoblastic cell lines: HBL-all, MOLT4, and Jurkat. Specific transcripts were not found in Jurkat cells. Two different bands could be observed: one of the expected size of ~ 640 bp, and a smaller one of ~ 320 bp. The control RT-PCR for β-actin is also shown below.

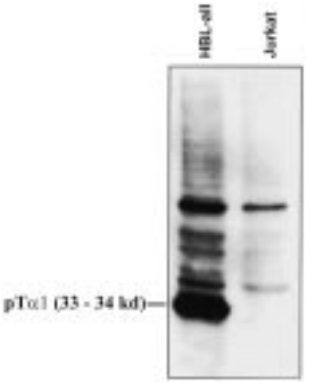

Figure 4. Immunoprecipitation of pTα1 in the human acute lymphoblastic cell lines HBL-all and Jurkat.

entire exon 2 is eliminated. The donor splice site of exon 1 and the acceptor splice site of exon 3 are used to yield pTα2. Therefore, the protein encoded by this cDNA isoform is predicted to consist of the signal peptide followed by the first three aa of the extracellular domain and finally, just following the connecting peptide which is necessary for the binding to the TCRβ chain, the transmembrane domain and the cytoplasmic tail. All these deduced domains are equal to those of pTα1. The expected molecular mass of pTα2 is of 18.4 kDa. In contrast to pTα1, which contains one N-glycosylation site in humans and two in mice, pTα2 has no potential N-glycosylation site. Both pTα1 N-glycosylation sites are encoded by exon 2. Nevertheless numerous serines and threonines, which are potential targets for O-glycosylation, are present in pTα1 and pTα2. Therefore, it is possible that the two pTα splice forms are O-glycosylated, like many cell membrane glycoproteins (for instance CD8 or IL-2 are O-glycosylated or O-glycan sialylated, respectively [15]). As no consensus sequence(s) for O-glycosylation sites could be defined up to now, further analyses are needed to establish possible glycosylations.

2.5 Immunoprecipitation of the human pTα protein

The human cell lines HPB-ALL and Jurkat, which express and do not express the two pTα isoforms, respectively, were labeled with [^{3}H]leucine. Lysates were immunoprecipitated with R225 antibodies. These polyclonal rabbit antibodies were generated against the complete extracellular domain of murine pTα including the connecting peptide. The antibodies recognize the protein generated *in vitro* from human pTα1 cDNA by an eukaryote-coupled transcription/translation system supplemented with dog pancreas microsomes (data not shown). Precipitates were analyzed by SDS-PAGE. A 33–34-kDa protein could be detected in precipitates from HPB-ALL but not from Jurkat cells (Fig. 4). This protein is likely to correspond to pTα1. The same results were obtained under reducing and nonreducing conditions and suggest that in HPB-ALL cells pTα1 is not linked to another protein. The human pTα2 isoform could not be detected in these experiments. It is possible, however, that the R225 antibodies do not recognize this form. In contrast to the Ig-like domain, which shows 85 % homology between mice and humans and is only present in pTα1, only 55 % homology exists between the murine and human 20 aa extracellular part which is shared by the two pTα isoforms. Thus, the R225 antibodies may not bind to the connecting peptide (compared to the Ig-like domain) of human pTα.

We were unable to precipitate pTα in biotinylated surface HPB-ALL cell extracts. In this cell line, which expresses the TCRαβ, pTα should not be expressed within a pre-TCR complex on the cell surface.

2.6 An additional pTα heterodimer in mice

Previous RT-PCR studies on pTα expression revealed two pTα forms in the murine thymus [16]. Since a corresponding smaller size mRNA could not be detected by Northern blotting we concluded that it was a PCR artifact. However, recently Barder et al. [17] reported the expression of mRNA corresponding to the two pTα isoforms. Prior sequence analysis had revealed that the murine splicing variant had exactly the same organization as human pTα [16].

To analyze whether pTα2 protein could be detected cell surface biotinylation was performed with the SCB29 cell

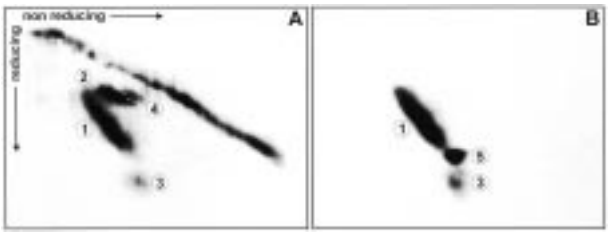

Figure 5. F23.1 (anti-Vβ8 monoclonal antibody) immunoprecipitates of SCB29 cell lysates analyzed by nonreducing/reducing SDS-PAGE. (A) Blots from surface biotinylated cells. 1: pTα1, 2: TCRβ (pTα1 associated); 3: a 20-kDa covalently TCRβ-linked protein, which may represent pTα2; 4: TCRβ (associated with the 20-kDa, TCRβ-linked protein). No other covalently TCRβ-linked protein could be detected. (B) The blot shown in (A) was destained and probed with the polyclonal rabbit anti-murine pTα antiserum R225. Spots 1 and 3 correspond to spots 1 and 3 seen in blot A, representing pTα1 and the putative pTα2, respectively. An additional spot could be detected by the pTα-specific antibody. As this protein (spot number 5) could not be detected by cell surface protein biotinylation, it probably represents an intermediate, intracellular pTα protein with incomplete posttranscriptional modifications.

line [18]. After cell lysis, pre-TCR complexes were immunoprecipitated with an anti-TCRβ antibody (F23.1) and analyzed by two-dimensional, reducing and nonreducing, diagonal gel electrophoresis. Gels were blotted and membranes labeled with streptavidin-horseradish peroxidase (HRP) to detect all precipitated proteins. Subsequently, the membrane was reprobed with a pTα-specific polyclonal antiserum (R225). Using the anti-TCRβ antibody two complexes could be precipitated (Fig. 5A): the first, representing the major complex, consisted of the TCRβ chain and the originally described, full-length pTα (pTα1). The second, weakly detectable complex consisted of a protein with the same size as TCRβ, linked to a second protein of 20 kDa (Fig. 5B). This latter protein was recognized by the polyclonal pTα antiserum R225. Since without glycosylation pTα could have an apparent molecular weight of 9 kDa we cannot be certain that a pTα2 protein is indeed associated with the TCRβ chain as has been claimed by Barder et al. [17]. The second heterodimer could also represent a degradation product of the pTα/TCRβ heterodimer.

3 Concluding remarks

Recently, Irving et al. [19] have shown that thymocytes expressing a pre-TCR lacking the extracellular Ig-like domains of both pTα as well as TCRβ develop normally. They generated $RAG^{-/-}$ transgenic mice expressing a truncated form of the TCRβ and the pTα chain, which had their (normal) extracellular domain replaced by a "flag" or "myc" epitope, but still had the cysteine residue necessary for heterodimerization. Thymocyte development could be restored in these transgenic mice. The truncated pTα construct used to generate these transgenic mice is very similar to the variant pTα isoform found in mice and humans but these mice obviously contained also endogenous pTα. Nevertheless, these results were interpreted to indicate that pre-TCR complex without extracellular Ig-like domains could be sufficient to drive the transition from the double-negative to the double-positive stage without binding to an extracellular ligand. Irving et al. [19] observed that in transgenic $RAG^{-/-}$ mice, that expressed only the truncated TCRβ Myc transgene, the modified pre-TCR (composed by the truncated β chain and the endogenous pre TCRα chain) was inefficiently expressed on the cell surface. Based on these results they proposed that only a symmetrical pre-TCR complex can be stable or properly assembled. Following this idea, a pre-TCR complex with pTα2 should not be stable. For the moment we can only speculate about the possible role of this differential splicing form. It is possible that pTα2 could compete with pTα1 for CD3 and TCRβ binding, forming an inactive or differentially signaling complex, thus providing a regulatory element in pre-TCR assembly.

4 Materials and methods

4.1 Cell lines and tissues

Three human acute lymphoblastic leukemia cell lines, kindly provided by Dr. MacIntyre (Hôpital Necker, Paris, France), were used for our experiments: the mature, TCR^+ and $CD3^+$ T cell lines HBL-all and Jurkat, and the γδ T cell line MOLT4 [20, 21]. These cell lines were cultured in RPMI 1640/10 % FCS (Gibco-BRL, Gaithersberg, MD). The immature, pre-TCR-positive T cell line SCB29 [1] was cultured in IMDM/10 % FCS (Gibco). A thymus from a 3-month-old child was purchased from Hôpital Saint-Louis (Paris, France).

4.2 Isolation, mapping and sequencing of genomic DNA

High-density colony filters corresponding to a cosmid library of human chromosome 6 (no. 109 L4/FS6; Reference Library Database, Max Planck Institute for Molecular Genetics, Berlin; [7]) were prehybridized overnight at 42 °C in 50 % formamide, 4 × SSC, 50 mM sodium phosphate (pH 6.8–7.2), 1 mM EDTA (pH 8.0), 10 % dextran sulfate, 1 % SDS, 50 μg/ml denatured salmon sperm DNA and 10× Denhart's solution [22]. Hybridization was

performed in the same solution and under the same conditions with an [α-^{32}P]dCTP random priming labeled (High-Prime, Boehringer Mannheim), human pTα cDNA probe [5]. For detection of positive clones, filters were washed three times in 0.1 % SSC at 65 °C and exposed to an X-ray film (Kodak). The pTα cosmids-containing bacterial clones DHR-5α-MCR were supplied by the Max Planck Institute for Molecular Genetics. Cosmid DNA was purified according to Birnboim and Doly [23].

DNA inserts were mapped by Southern blotting: endonuclease-digested cosmid DNA samples were subjected to electrophoresis and transferred with 0.4 M NaOH to Hybond N+ membranes (Amersham). Filters were prehybridized and hybridized in 5 × SSC, 5× Denhart 1 % SDS at 65 °C. Sequences of the human cDNA corresponding to the different pTα domains (leader peptide, Ig-like domain, and cytoplasmic tail) were used as probes for subsequent ^{32}P labeling. For rehybridizations probes were removed by incubating membranes for 10 min at 95 °C in 0.5 % SDS. DNA inserts were cut by enzyme restriction and subcloned into a Bluescript-KS$^+$ vector (Stratagene, La Jolla, CA). DNA subclones were sequenced with an ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Warrington, GB) and by automated sequencing ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems).

4.3 RT-PCR

Total RNA was isolated using the RNeasy Mini kit (Qiagen, Hilden, Germany). Reverse transcription was carried out using Superscript II reverse transcriptase kit (Gibco). Subsequent PCR was carried out in a total volume of 50 μl, containing 1 mM deoxynucleoside triphosphates (dNTP), 0.5 μM of each primer, 5 μl 10× buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.3, 15 mM $MgCl_2$ and 0.01 % (w/v) gelatin, in a volume of 1.5 ml), 2 μl cDNA from the RT step, and 2 U AmpliTaq® DNA polymerase, LD (PE Applied Biosystems).

After a 3-min denaturation step at 94 °C, 40 cycles consisting of 45 s at 94 °C, 1 min at a primer-specific annealing temperature (T_A), and 1 min at 72 °C were performed in a Gene Amp PCR System 9700 thermal cycler (PE Applied Biosystems). Amplified DNA fragments were analyzed on a 2 % agarose gel in 1× TAE buffer. The PCR primers used for human pTα ($T_A = 60$ °C) were CTGCAGCTGGGTCCTGCCTC and AGTCTCCGTGGCCGGGTGCA. Primers for human β-actin ($T_A = 55$ °C) were ACACTGTGCCCATCTACGAGGG and ATCATGGAGTTGAAGGTAGTTTCG.

4.4 Protein analysis

L-[4,5-^{3}H]-leucine metabolic and cell surface biotinylation were performed as described previously [24]. Subsequently, cells were lysed for 1 h in Triton lysis buffer (20 mM Tris/HCl, 150 mM NaCl, 2 % Triton X-100, 1 mM $MgCl_2$, pH 8) or digitonin lysis buffer (10 mM triethanolamine, 150 mM NaCl, 1 % digitonin, 1 mM EDTA) supplemented with 5 mg/ml aprotinin, 5 mg leupeptin, 100 mM PMSF and 10 mM iodoacetamide. Nuclei were removed by a 10 min centrifugation at 12 000 × g at 4 °C. The remaining supernatant was precleared four times by a 2–6-h incubation with normal rabbit or mouse serum and protein G-Sepharose CL-4B (pG; Pharmacia, Uppsala, Sweden) in Triton or digitonin lysis buffer.

Proteins were immunoprecipitated by a 3-h incubation at 4 °C with pG-coupled R225 polyclonal antiserum (rabbit anti-murine pTα extracellular domain; [25]) or F23.1 monoclonal antibody (mouse anti-murine TCRβ-Vβ8). Unbound proteins were removed by washing four times with 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 twice with NET-T (NET-TON with 0.15 M NaCl and without ovalbumin). Proteins were eluted from beads with reducing or nonreducing sample buffer (10 % glycerol, 2.3 % SDS, 62.5 mM Tris-HCl, pH 6.8, 0.05 % w/v bromophenol blue ± 10 mM mercaptoethanol) by boiling for 5 min and subsequent addition of iodoacetamide (60 mM final concentration).

Proteins were run in a one-dimensional 12 % polyacrylamide gel or in two-dimensional diagonal SDS-polyacrylamide gels (nonreducing 8 % polyacrylamide gel in the first and reducing 12 % polyacrylamide gel in the second dimension) as described previously [1]. For visualization of radioactive proteins gels were fixed, incubated in Amplify solution (Amersham), dried and analyzed by autoradiography.

After electrophoresis, samples were transferred onto polyvinylidene difluoride membranes by semi-dry blotting. Membranes were blocked in the following solution: PBS, 4 % Tween 20, 2 % fetal calf serum 10 % non-fatty milk powder. Blots were incubated with streptavidin-HRP conjugate (Southern Biotechnology, Birmingham, AL) for 30 min, at room temperature in PBS 4 % Tween 20. HRP was detected by incubation with ECL detection solution (Amersham) and exposure to an X-ray film (Kodak). Blots were destained by incubation in PBS/0.1 % sodium azide for reprobing. Subsequently, blots were incubated in PBS 4 % Tween 20 with the pTα-specific R225 rabbit antiserum (1/1000), washed three times in PBS/4 % Tween 20 and incubated for 1 h with HRP goat anti rabbit IgG (1:20 000 dilution; Southern Biotechnology). HRP detection was performed as described above.

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