Molecular Cloning of pTAC12 an Alternative Splicing Product of the CD3γ Chain as a Component of the Pre-T Cell Antigen-Receptor Complex*

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Kan Takase‡, Yasushi Okazaki‡, Keisuke Wakizaka‡, Andrej Shevchenko§, Matthias Mann§, and Takashi Saito¶

From the ‡Department of Molecular Genetics, Chiba University Graduate School of Medicine, 1-8-1 Inohana, Chu-o-ku, Chiba 260-8670, Japan, and the §Protein and Peptide Group, European Molecular Biology Laboratory, Meyerhofstrasse 1, 69117 Heiderberg, Germany

We have reported that a 12-kDa molecule (pTAC12 as a pre-T cell receptor (TCR)-associated chain) was associated as a dimer with the pre-TCR complex as well as the clonotype-independent CD3 complex on the cell surface of immature thymocytes. We now report by protein sequence and molecular cloning that pTAC12 is an alternatively spliced product of the CD3γ chain lacking exon containing the transmembrane region. The transcript of pTAC12 is expressed in most T cell lineages and parallels the expression of CD3γ. However, the pTAC12 protein is expressed on the cell surface of immature thymocytes but not mature T cells, despite the fact that mature T cells express a low level of pTAC12 in association with the TCR complex within the cells. These results indicate that pTAC12 may play a special role for the transport/expression and assembly of the pre-TCR-CD3 complex as well as the clonotype-independent CD3 complex in immature thymocytes.

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Thymocyte differentiation generates a functional repertoire of T cells competent for carrying out the immune response. The mechanism of thymocyte selection through the T cell receptor (TCR) has been extensively analyzed, whereas the regulation of the development prior to the expression of the TCRγδ dimer is still largely unknown. Surface expression of the TCRγ chain regulates a crucial step of early T cell development from CD4+CD8– (double negative) to CD4+CD8+ (double positive) stages in TCRγδ-expressing T cells (1). It was demonstrated that these immature thymocytes express the pre-TCR complex, which is composed of the pre-TCRα chain (pTα) and the TCRβ chain in association with the CD3 complex, before the TCRα gene is rearranged and expressed (2, 3). Analyses of TCRβ- (4) and pTα-deficient mice (5) have revealed a critical role of the pre-TCR complex in the regulation of early thymocyte development. The structure of pre-TCR resembles that of pre-B cell antigen receptor, which is composed of the μ-heavy chain, λ5, and VpreB. Although pTα may be equivalent to λ5 because of the fact that both consist of an Ig-like constant domain, a “VpreB” molecule equivalent to VpreB has not been identified yet. Because it has been shown that CD3ζ and ZAP-70 are phosphorylated after stimulation by cross-linking of the pre-TCR complex with anti-CD3ε mAb in a cell line (6) and that Lck plays an important role for thymic development (7–9), signaling through the pre-TCR complex might be similar to that in mature T cells. However, because the physical association between the pre-TCR complex and CD3ζ is very weak (10–13) and thymocytes differentiate to the double positive stage in CD3ζ-deficient mice (14–17), CD3ζ may not be essential for early T cell development. Differential assembly of the components within the pre-TCR complex may suggest a unique regulation of its assembly and expression.

In addition to the pre-TCR-CD3 complex, the clonotype-independent CD3 (CIC) complex lacking TCRα and β chains is present on the cell surface of immature thymocytes (18). The CIC complex, which might also be associated with unknown receptors, was found to be associated with the molecular chaperone calnexin (19). We extended this finding to the pre-TCR complex by analyzing an immature thymocyte cell line, KK, which expresses pTα-β dimer, and found that calnexin is also associated with the pre-TCR complex (10). In addition to calnexin, we found that a certain 12-kDa molecule is associated with both pre-TCR and CIC complexes as a homodimer and also that this association is much stronger than that between CD3ζ and the pre-TCR complex (10).

The present study was performed to identify this unknown 12-kDa molecule, referred to as pTAC12 (a pre-TCR-associated chain), which is associated with pre-TCR and CIC complexes. Protein purification and microsequencing revealed that pTAC12 is an alternatively spliced product of the CD3γ chain, as a component of the pre-TCR-CD3 complex. The unique structure and features of pTAC12 are presented, and the possible function of pTAC12 is discussed. Our observations may help to understand this unique receptor complex, its signal transduction, and the development of immature thymocytes.

EXPERIMENTAL PROCEDURES

Animals—C57BL/6 mice were purchased from Japan CLEA Inc. (Tokyo, Japan). RAG-2-deficient mice were kindly provided by Dr. F. Alt (Harvard Medical School, Boston, MA).

Cell Lines and Antibodies—The immature thymocyte cell line KKF was derived from Gross’ leukemia virus-infected BALB/c thymocytes and the expressed pre-TCR complex as described previously (10, 11). The SCB29 cell line, derived from severe combined immunodeficient mouse thymocytes and also expressing pre-TCR, was kindly provided by Dr. H. von Boehmer (Necker Institute, Paris, France) (2, 3). DO11-10, a chicken ovalbumin-specific murine T cell hybridoma; 2C-1, a mouse T cell clone specific for KLH; MA5, a mouse T cell hybridoma; and DAP-3, a mouse fibroblast T cell clone were previously described. Anti-mouse CD3ε mAb (145-2C11) and rabbit anti-mouse CD3γ Ab (no. 125) (20) were kindly provided by Dr. J. Bluestone (University of Chicago, Chicago, IL) and Dr. J. S. Bonifacino (NIH, Bethesda, MD).
Truncated CD3γ within pre-TCR Complex

respectively. Protein Microsequencing—Protein in a reducing and nonreducing two-dimensional diagonal gel was visualized by silver staining, excised from the gel, and digested in-gel with trypsin as described previously (21). The unseparated pool of tryptic peptides was desalted and concentrated using a microcapillary containing approximately 100 nl of PO-ROS R2 reversed phase materials (PerSeptive Biosystems Inc., Framingham, MA) and sequenced by nano-electrospray tandem mass spectrometry as described (22). Analysis was performed on an API III triple quadrupole (Perkin Elmer Sciei Instruments, Toronto, Canada). A nonredundant protein data base containing more than 250,000 entries was used (GS Sander, European Bioinformatics Institute, Hinxton Hall, United Kingdom).

3'-Rapid Amplification of cDNA End—Using total RNA prepared from KKF cells with guanidine thiocyanate, cDNA was prepared with a Superscript II kit (Life Technologies, Inc.) and oligo(dT) primer QT (23). cDNA was then amplified by two-step PCR. The primers for the first PCR recognized the 5'-upstream sequence proximal to the identified peptide sequences of CD3γ (5'-GCTCTCTTCCGGTGTACATCTCT-3') and Q9 (23), which is complementary to Q9. The primers for the second PCR were designed to recognize the inside sequence of the first PCR primers (5'-ATGGATCCGACATGGAGCAGAG-GAAGGTC-3' and 5'-GCATCGAGCCAGCTGTCCATTCTCCTCAG-3'), and a downstream primer designed for the specific amplification of pTAC12 recognized the sequence including both parts of exon 3 and exon 5 of the CD3γ gene (5'-CTTGCCAGCTGTCCATTCTCCTCAG-3'). This led us to assume that pTAC12 is expressed on the cell surface of T cell line, C5.1, expressing little CD3γ complex on the cell surface (10).

Reverse Transcripase-PCR—Total RNA from cell lines, cell suspensions, and various tissues was prepared with guanidine thiocyanate. The cDNA was prepared with random hexamer primers and reverse transcribed with a Superscript II kit (Life Technologies, Inc.). cDNA diluted in water was then used for PCR amplification. Primers used for CD3γ recognized sequences including the initiation and termination codons of the CD3γ gene (5'-ATGGATCCGACATGGAGCAGAG-GAAGGTC-3' and 5'-GCATCGAGCCAGCTGTCCATTCTCCTCAG-3'), and a downstream primer designed for the specific amplification of pTAC12 recognize the sequence including both parts of exon 3 and exon 5 of the CD3γ gene (5'-CTTGCCAGCTGTCCATTCTCCTCAG-3'). The specific amplification of pTAC12, but not the intact CD3γ, with this pair of primers was confirmed by using the plasmid containing either of their cDNA (data not shown). PCR amplification was performed for 30 cycles with an annealing temperature of 50 °C for CD3γ and 47 °C for pTAC12.

Cell Surface Biotinylation, Immunoprecipitation, SDS-PAGE Analysis, and Western Blotting—Cell surface biotinylation, immunoprecipitation, SDS-PAGE, and Western blotting were performed as described previously (10). For detection of pTAC12, 1 × 10^6 T cells/sample were used for biotinylation and Western blotting. T cells were solubilized in a buffer containing 1% Brij 97. For immunoprecipitation, 10 μg of 2C11 or 10 μl of anti-CD3γ serum was used. For immunoblotting, a 1:5000 dilution of anti-CD3γ Ab was used. Reducing and nonreducing two-dimensional SDS-PAGE analysis was performed by using 16% acrylamide gel for the first dimension under nonreducing conditions and 18% gel for the second dimension under reducing conditions.

RESULTS

pTAC12 Is an Alternatively Spliced Product of the CD3γ Chain—To identify pTAC12, we purified this protein from 1.4 × 10^10 KKF cells, which we identified originally as a cell line expressing the pTα-β/CD3 complex on the cell surface (10). The position corresponding to pTAC12 was excised from a reducing and nonreducing two-dimensional SDS-PAGE gel, and the protein was sequenced by the nano-electrospray tandem mass spectrometry sequencing method (22). We obtained a 10-amino acid sequence that was identical to the extracellular (EC) domain of the CD3γ chain (Fig. 1). Because the molecular mass of the CD3γ chain and pTAC12 was approximately 18 and 12 kDa, respectively, it was apparent that pTAC12 was not identical to CD3γ. This led us to assume that pTAC12 is an alternatively spliced form of the CD3γ chain. Because the sequence we obtained was close to the N terminus of the CD3γ chain (amino acid numbers 7–16), we performed 3'-rapid amplification of cDNA end to isolate the full-length cDNA of pTAC12. Indeed, we obtained two distinct products approximately 670 and 540 bp in size. These two species were subcloned and sequenced. The longer form was found to correspond to the intact CD3γ, and the shorter one was identical to the CD3γ chain except for the lack of exon 4, which encodes the region containing the transmembrane domain (Fig. 1). It was noted that two cysteine residues in the membrane-proximal EC domain were also deleted in pTAC12. From this sequence analysis, it was determined that pTAC12 protein consists of 138 amino acids, and its molecular mass was 15.5 kDa, whereas the CD3γ chain has 182 amino acids and a molecular mass of 20.2 kDa. Without the signal peptides, pTAC12 protein is composed of 116 amino acids with a molecular mass of 13.1 kDa as compared with 161 amino acids and a molecular mass of 17.9 kDa for CD3γ.

Anti-CD3γ Ab Reacts with pTAC12—To confirm that pTAC12 is an alternatively spliced product of the CD3γ chain, the reactivity of pTAC12 with anti-CD3γ Ab was investigated. KKF cells were surface-biotinylated, and the cell lysates were immunoprecipitated with anti-CD3ε mAb and resolved on reducing and nonreducing two-dimensional SDS-PAGE, followed by either the detection of biotinylated protein or by immunoblotting with anti-CD3γ Ab. As the top panel in Fig. 2 shows, surface-biotinylated pTAC12, which was co-precipitated with the CD3 complex and observed as an off-diagonal dimer spot, indeed reacted with anti-CD3γ Ab. When the lysate was immunoprecipitated and blotted with anti-CD3γ Ab, the pTAC12 dimer was also detected, confirming that pTAC12 and CD3γ had the same reactivity to anti-CD3γ Ab (data not shown).

These results demonstrate that pTAC12 is an alternatively spliced product of the CD3γ chain and functions as a constituent of the pre-TCR-CD3 complex.

pTAC12 Is Expressed in All Lineages of T Cells—We previously showed that pTAC12 is expressed on the cell surface of immature thymocytes (KKF cells and severe combined immuno-deficient thymocytes) but not T cell hybridoma cells (10). Therefore, we analyzed whether the expression of pTAC12 is restricted to immature thymocytes. We investigated the mRNA expression of CD3γ and pTAC12 in several types of cells and tissues by reverse transcriptase-PCR. We utilized two pairs of primers: one for the detection of both CD3γ and pTAC12 and the other specific for pTAC12. With the pTAC12-specific primers, we found that pTAC12 mRNA was expressed in all lineages of T cells examined without a significant difference in expression level but was not expressed in other types of cells such as L cells (Fig. 3) or other tissues such as brain (data not shown). The expression of pTAC12 was parallel to that of CD3γ. Consistent with this notion, a variant immature thymocyte cell line, C5.1, expressing little CD3γ transcript, if any, also did not express any detectable level of pTAC12 mRNA (data not shown). By using the other pair of primers corresponding to the 5' and 3'-ends of the CD3γ exons, two bands (574 and 442 bp) were observed (Fig. 3, top). The longer product corresponded to the intact CD3γ and the shorter one to pTAC12. The expression level of pTAC12 was weak and approximately one-tenth of that of CD3γ, and the ratio of pTAC12 to CD3γ did not vary among these T cells.

These results indicate that the transcript of pTAC12 is expressed in most T cells regardless of the developmental stage and that it is parallel to the expression of CD3γ.

Cell Surface Expression of pTAC12 Is Restricted to Immature Thymocytes—To analyze the cell surface and intracellular expression of the pTAC12 protein in mature T cells, we performed surface biotinylation and Western blot analysis of mature T cells. Splenic T cells and an antigen-specific T cell clone, 23-1-8, were surface biotinylated, and the cell lysates were immunoprecipitated with anti-CD3ε mAb and analyzed for biotinylated
surface protein or immunoblotted with anti-CD3γ Ab. As shown in the middle and bottom panels of Fig. 2, despite the fact that pTAC12 was not detectable on the cell surface of either splenic T cells or T cell clones, a low level of the intracellular pTAC12 protein was detected by blotting with anti-CD3γ Ab in the immunoprecipitate with anti-CD3ε mAb. These results demonstrate that in mature T cells, pTAC12 is produced and expressed intracellularly and that a part of the protein is associated with the TCRγCD3 complex within the cells, but such a complex containing pTAC12 cannot be expressed on the cell surface of mature T cells. Collectively, our results indicate that the cell surface expression of pTAC12 is restricted to immature thymocytes.

DISCUSSION

In thymocyte development, pre-TCR consisting of pTα and TCRβ plays a crucial role in promoting the differentiation of double negative thymocytes into double positive cells and their proliferation. Although it has been shown that the pre-TCR complex induces phosphorylation of CD3ζ and ZAP-70 and Lck plays an essential role in signaling through the complex, the precise structure of and signaling pathway through the pre-TCR complex remain to be elucidated. For the structure of the pre-TCR complex, in addition to calnexin expressed on the CIC complex and the pre-TCR complex (10, 19), we found that a new 12-kDa dimer, pTAC12, was also associated with the pre-TCR and CIC complexes on the cell surface of immature thymocytes (10). In the present study, we determined the protein sequence and succeeded in cloning the pTAC12 cDNA. pTAC12 was found to be an alternatively spliced product of CD3γ lacking exon 4.

The CD3γ chain is noncovalently associated directly with CD3ε and indirectly with CD3δ and constitutes the CD3 complex as γεδ (24). The CD3γ gene, consisting of seven exons, is highly homologous to the CD3δ gene (25), and their transcription seems to be controlled by common regulatory elements (26). However, it has been suggested that γ and δ have a distinct function. The study of CD3δ-deficient mice showed that CD3δ is not required for early thymocyte development or γδ T cell differentiation (27, 28). In contrast, CD3γ deficiency blocks the transition from the double negative to double positive stages that is controlled by the pre-TCR complex (29). The crucial role of CD3γ for the cell surface expression of the TCR complex remain to be elucidated. As for the structure of the pre-TCR complex, in addition to calnexin expressed on the CIC complex and the pre-TCR complex (10, 19), we found that a new 12-kDa dimer, pTAC12, was also associated with the pre-TCR and CIC complexes on the cell surface of immature thymocytes (10). In the present study, we determined the protein sequence and succeeded in cloning the pTAC12 cDNA. pTAC12 was found to be an alternatively spliced product of CD3γ lacking exon 4.
TCR\(\gamma\) complex has been demonstrated by the CD3\(\gamma\)-deficient human Jurkat cell line (30). These studies revealed that the EC region of CD3\(\gamma\) is essential for the surface expression of the TCR complex (31). Because pTAC12 lacks exon 4 containing the transmembrane region and possesses most of the EC domain of CD3\(\gamma\), pTAC12 may play a role in the surface expression of pre-TCR by assembling with the complex.

The assembly of the pre-TCR-CD3 complex is considerably weaker than that of the TCR-CD3 complex of mature T cells, particularly for the association with CD3\(\zeta\) (10–13, 32). Thus the pre-TCR complex may have a structure and stability distinct from the TCR complex of mature T cells, and calnexin may associate with such unique or unstable complexes. Because calnexin is usually retained within ER, its expression on the cell surface may occur only under the special condition of such a unique assembly of the receptor complex in immature thymocytes. The expression of pTAC12 on the cell surface may be controlled by a similar regulation used for calnexin. The pre-TCR complex may have a distinctly unstable structure compared with the TCR complex on mature T cells. First, the pTac-\(\alpha\)-\(\beta\) dimer may have a different configuration from the TCR complex of mature T cells even in the presence of VpreT, and second, the pre-TCR complex has a very weak association with CD3\(\beta\). This unstable structure might require pTAC12 and/or calnexin. Our observations suggest that pTAC12 may have a special function for the cell surface expression of pre-TCR and CIC complexes. This may explain why mature T cells cannot express pTAC12 and calnexin on the cell surface despite the fact that both proteins are present intracellularly.

The precise mechanism of the association of pTAC12 with the pre-TCR complex still has to be determined. pTAC12 exists as a disulfide-linked homodimer, and its sequence lacks two transmembrane-proximal cysteine residues; it has only two cysteines in the EC region, which constitute an Ig-like domain in the intact CD3\(\gamma\) chain. This suggests that pTAC12 might use these cysteines for the homodimer formation. If this is the case, pTAC12 may have a different structure from CD3\(\gamma\), and the structure of the Ig-like EC domain may be altered. From this notion and also from the structural basis, pTAC12 might be secreted and function as a kind of cytokine to regulate T cell differentiation. The observation that pre-B cells are able to...
secrete a variety of proteins including pre-B cell antigen receptor and VpreB is consistent with this assumption (33).

This is the first report to describe an alternative splicing product of CD3\(\gamma\). The human CD3\(\gamma\) mRNA exists in two forms in human T cells, and they were found to be alternative splicing products of CD3\(\gamma\). A putative protein would lack the transmembrane region, similar to our finding in mouse CD3\(\gamma\), and was assumed to be secreted or associated with surface molecules (34). Although an alternative splicing product of CD3\(\gamma\) was not detected in the mouse system (data not shown), the alternative splicing product of CD3\(\gamma\) in humans might have also some specific function similar to that of pTAC12 in the mouse system.

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