PJA-BP expression and TCR δ deletion during human T cell differentiation

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Abstract

Recombination of δRec to ψJα will delete the TCR δ gene, which is thought to play an important role in the bifurcation of the TCR αβ versus TCR γδ differentiation lineages. We recently detected a DNA-binding protein in human thymocytes, the so-called PJA-BP, which recognizes the ψJα gene segment and might be one of the factors involved in the regulation of preferential δRec–ψJα rearrangements. We now investigate PJA-BP expression and its correlation with TCR δ gene deletion in thymocytes. Our electrophoretic mobility shift assay experiments showed that the PJA-BP is evolutionary conserved in human, murine and simian thymocytes. Using a large series of human hematopoietic malignancies (n = 30), we conclude that PJA-BP expression is thymocyte specific and seems to be restricted to thymocytes committed to the TCR αβ lineage. Analysis of seven well-defined human thymocyte subpopulations showed that preferential δRec–ψJα rearrangements as well as PJA-BP expression can be detected from the immature CD34–/CD1+/CD3–/CD4+/CD8α+β– thymocyte differentiation stage onwards. These experiments indicate that expression of PJA-BP in human thymocytes starts simultaneously with preferential δRec to ψJα rearrangements, which supports our hypothesis that PJA-BP is one of the factors involved in the preferential recombination of δRec to ψJα.

Introduction

The TCR δ gene complex is flanked by the TCR δ-deleting elements δRec and ψJα (1,2), which are assumed to delete the TCR δ gene before Vα–Jα rearrangement (3). The non-productive δRec–ψJα recombination occurs at a high frequency in both human and murine thymocytes (1,2,4,5). However, little is known about the cell stage at which this rearrangement is initiated during human T cell differentiation and also the mechanisms that regulate this preferential rearrangement are unknown.

We recently detected a DNA-binding protein of ~180 kDa, the so-called ψJα-binding protein (PJA-BP), which recognizes the ψJα gene segment (6). Preliminary results on the expression pattern of PJA-BP in some human cell lines suggested that PJA-BP expression might be correlated with TCR δ deletion or consecutive Vα–Jα rearrangements. Based on our previous data, we postulated that the PJA-BP might be one of the factors involved in the preferential recombination of δRec to ψJα (6).

In this study we characterized the expression pattern of the PJA-BP in different species and in human hematopoietic malignancies. Furthermore, we investigated the correlation between PJA-BP expression and TCR δ gene deletion in seven well-defined human thymocyte subpopulations.

Methods

Cell samples

Human thymocytes were obtained from postnatal thymus samples of children undergoing cardiac surgery at the age...
of 22 days to 2 years, as described previously (6). All human thymus samples were obtained with the approval of the Medical Ethics Committee of the Erasmus University Rotterdam/University Hospital Rotterdam, Rotterdam, The Netherlands.

Murine thymocytes were obtained from 6-week-old C57Bl/6 mice. Simian thymocytes were obtained from an adult Macaque.

Patient cell samples were obtained from 30 patients with a hematopoietic malignancy: 10 T cell acute lymphoblastic leukemias (ALL), including six TCRαβ− T-ALL, two TCRαβ+ T-ALL and two TCRγδ+ T-ALL; five mature T cell leukemias, including three TCRαβ+ T cell large granular lymphocyte leukemias (T-LGL) and two TCRαβ+ Sézary syndromes; five precursor-B-ALL; five B cell chronic lymphocytic leukemias (B-CLL); and five acute myeloid leukemias (AML). Peripheral blood mononuclear cells (PBMC) were isolated from the patients and from healthy volunteers by Ficoll-paque (density 1.077 g/ml; Pharmacia, Uppsala, Sweden) density centrifugation.

Preparation of thymocyte subpopulations

To isolate the most immature thymocyte subpopulations, Lymphoprep (density 1.077 g/ml; Nycomed Pharma, Oslo, Norway) density centrifugation was performed on thymocyte suspensions. The CD34+ population was then enriched using the QBEND/10 kit (Miltenyi Biotec, Auburn, CA) following the manufacturer’s instructions. Subsequently, the CD34+/CD1− subpopulation (fraction 1) and the CD34+/CD1+ subpopulation (fraction 2) were sorted with a FACStar Plus (Becton Dickinson, San Jose, CA) using CD34 (HPCA-2) and CD1a (T6-RD1) antibodies. The CD8+ subpopulation (fraction 4) was obtained by depletion of glycoporphin+ CD8+, CD27+, CD69+, CD19+ and CD3+ cells using the 10F7 MN, RPA-T8, CLB-3A12, Leu-23, CLB CD19 and SPV-T3b antibodies respectively, followed by depletion with magnetic beads coated with sheep anti-mouse Ig (Dynal, Oslo, Norway). Part of this subpopulation was further sorted for the CD3−CD4+/CD8− phenotype (fraction 3) with a FACStar Plus. Purity of the cell population was >97%.

Two additional subpopulations were obtained by depletion of CD8β+, CD27+ and CD69+ cells (fraction 5), or CD27+ and CD69+ cells (fraction 6) using the 2ST8-5H7, CLB-3A12 and Leu-23 antibodies and magnetic beads (Dynal). Bead-coated thymic cells of the latter fraction, which contained the more mature CD27+/CD69− thymocytes (fraction 7), were obtained by magnetic separation.

Electrophoretic mobility shift assay (EMSA)

Crude nuclear protein extract (2 µg) was incubated for 30 min at room temperature with the 32P-labeled human DNA probe TCRAPJ-C1 (Fig. 1A) as described previously (6). Furthermore, EMSA experiments were performed with an additional DNA probe (Mu1TCRAPJ; Fig. 1A) containing the murine ψJα gene segment (7,8).

To exclude proteolytic activity in our nuclear extracts, we checked the nuclear protein extracts for the presence of the ubiquitously expressed octamer binding factor Oct1. For this purpose we performed EMSA experiments, as described previously (9), using the double stranded oligonucleotide GAGGAGATTTGACCTCCACGGCACCTCC, which contains a consensus binding site for Oct1.

Southern blot analysis

DNA was isolated from the thymocyte subpopulations, digested with the restriction enzyme EcoRI, size fractionated in agarose gels (0.7%), and transferred to Nitran-13N nylon membranes (Schleicher & Schuell, Dassel, Germany) as previously described (10). Filters were hybridized with a 32P random-oligonucleotide-labeled TCRDRE probe (11).

RT-PCR

Total RNA was isolated using the method of Chomczynski and Sacchi (12). Northern blotting was performed as described previously (13) using a 32P end-labeled Cα probe. For RT-PCR reactions 1 µg of RNA was incubated at 70°C for 10 min in a thermal cycler (PE-Applied Biosystems, Forster City, CA). Following this initial cycle additional steps were performed at room temperature for 10 min, 60°C for 45 min and 99°C for 2 min in 20 µl reaction volume containing 20 mM Tris–HCl (pH 8.3), 50 mM KCl, 1 mM MgCl₂, 10 mM dithiothreitol, 5 µM random hexamer primers [pd(N)₆; Pharmacia, Uppsala, Sweden], 1 mM DNA polymerization mix (Pharmacia), 20 µl rRNAin (Promega, Madison, WI) and 200 µl SuperScript II RT ( Gibco/BRL Life technologies, Paisley, UK). The PCR analysis was performed using 1 µl of RT reaction mix and 10 pmol of specific oligonucleotide primers in 25 µl reaction volume containing 1×PEII buffer (PE-Applied Biosystems), 0.2 mM DNA polymerization mix (Pharmacia), 2.5 mM MgCl₂, and 0.5 U of AmpliTaqGold (PE-Applied Biosystems). The PCR reaction mixture was incubated at 94°C for 10 min in a thermal cycler (PE-Applied Biosystems). Following this initial cycle, denaturation, annealing and extension steps were performed for another 35 cycles at 94°C for 1 min, at 60°C for 1 min and at 72°C for 3 min respectively. The PCR products were blotted and hybridized with internal 32P end-labeled oligonucleotide probes as described previously (5). Oligonucleotide primers and probes used were: ABL sense, 5’-GACCCCAACATTTTCGTTGCACAC-3’; ABL antisense, 5’-ATGGTACCCAGGAGTGTTCTCTTG-3’; TCRα antisense, 5’-TCGGTAATAGGCAGCAG-3’. Sequences were obtained from EMBL databank accession nos G28237 for the human ABL gene, and HUA000658 through HUA000662 for the human TCRα gene segments.

Results and discussion

Expression of the PJA-BP in murine and simian thymocytes

We recently detected a DNA-binding protein in human thymocytes which recognizes the ψJα gene segment (6). We were interested to see whether this protein is evolutionary conserved. The human ψJα gene segment shows 80% homology with the murine ψJα gene segment (2). The major difference between the human and murine gene segments is the presence of a variable number of T nucleotides in the murine
Fig. 1. PJA-BP expression in human, simian and murine thymocytes. (A) Homology between the human and murine ψJα gene segments (2,7,14,15). The presented ψJα sequences start immediately downstream from the heptamer of the recombination signal sequence. The human (TCRAPJ-C1) and murine (MuTCRAPJ) DNA probes used in this study are indicated; the ψJα sequences in lower case characters are not present in the TCRAPJ-C1 probe. The human PJA-BP binding site is depicted in bold and the additional ATTAC core domain of the murine ψJα gene segment is underlined. EMSA of the human DNA probe TCRAPJ-C1 (B, left panel) or the murine DNA probe MuTCRAPJ (B, right panel) using crude nuclear extracts from human PBMC (lane 1), human thymocytes (lane 2), murine thymocytes (lane 3) and simian thymocytes (lane 4).

ψJα gene segment (2,7,14,15) (Fig. 1A). However, these nucleotides are not located in the major DNA-binding site of the PJA-BP (6). EMSA experiments using the human TCRAPJ-C1 DNA probe showed PJA-BP expression in nuclear extracts from murine thymocytes and weak PJA-BP expression in nuclear extracts from simian thymocytes (Fig. 1B, left panel).
Interestingly, we were not able to detect PJA-BP expression in nuclear extracts from normal human PBMC (Fig. 1B) nor in nuclear extracts from the E-rosette fraction of PBMC representing mature T lymphocytes (data not shown). These results and our previous data using several human hematopoietic cell lines (6) suggests that PJA-BP expression might be thymocyte specific.

We also cloned (and sequenced) the murine \( \psi J_\alpha \) DNA probe MuTCRAPJ (Fig. 1A). EMSA experiments using this DNA probe showed that the PJA-BP in nuclear extracts from human, murine and simian thymocytes can also bind to the murine \( \psi J_\alpha \) DNA probe (Fig. 1B, right panel). PJA-BP binding to the murine DNA probe appeared to be stronger than to the human DNA probe in all nuclear extracts tested (Fig. 1B). Nevertheless, nuclear extracts of human PBMC again did not show any PJA-BP expression.

To further investigate the difference in PJA-BP binding to the human and murine DNA probes, we performed competitive EMSA experiments (Fig. 2). Using nuclear extracts from human thymocytes, we showed that the labeled murine \( \psi J_\alpha \) DNA probe MuTCRAPJ could be competed completely with 10 ng of unlabeled MuTCRAPJ probe, but not with 100 ng of the unlabeled aspecific probe TCRAJ57 (6) (Fig. 2A). The unlabeled human TCRAJ-C1 probe could also compete the MuTCRAPJ probe completely (Fig. 2A). However, complete competition needed more unlabeled human TCRAJ-C1 probe (100 ng) than unlabeled MuTCRAPJ probe (10 ng). Comparable results were obtained when we repeated the experiments with nuclear extracts from murine thymocytes (Fig. 2B) or when we used labeled TCRAJ-C1 probe (data not shown). The stronger binding of PJA-BP to the MuTCRAPJ probe compared to the human TCRAJ-C1 probe can be explained by the presence of an additional TAAT/ATTA domain, necessary for PJA-BP binding (6), in the murine \( \psi J_\alpha \) sequence (underlined sequence in Fig. 1A).

The expression level of PJA-BP seems to be higher in murine thymocytes than in human thymocytes, because we had to add more unlabeled probe in our competition experiments when we used nuclear extracts from murine thymocytes (Fig. 2), despite the usage of identical amounts of human and murine nuclear extracts in the experiments.

We conclude from our EMSA experiments that PJA-BP is evolutionary conserved.

Expression of the PJA-BP in human hematopoietic malignancies

Our previous EMSA experiments with nuclear extracts from human hematopoietic cell lines showed that PJA-BP expression might be correlated with TCR \( \delta \) gene deletion and subsequent V\( \alpha \)-J\( \alpha \) rearrangements (6). In order to further investigate the correlation between TCR \( \delta \) gene deletion and the expression pattern of the PJA-BP, we performed EMSA experiments using crude nuclear extracts from 30 different human hematopoietic malignancies (representative examples are given in Fig. 3).

We were able to detect weak PJA-BP expression in five out of six TCR \( ^{+, \delta} \) T-ALL tested and in the two TCR \( \alpha\beta^{+, \delta} \) T-ALL tested. The weak bands could be competed with unlabeled
TCRAPJ-C1 but not with unlabeled TCRAJ57 (data not shown). However, the two TCR \( \gamma \delta \) T-ALL tested were negative for PJA-BP expression. In contrast to our previous data (6), no full correlation was found between PJA-BP expression and TCR \( \delta \) gene deletion in these immature T cell malignancies, because PJA-BP expression was observed in one patient with both TCR \( \delta \) genes in germline configuration and in one patient with two rearranged TCR \( \delta \) genes. Nevertheless, the absence of PJA-BP expression in TCR \( \gamma \delta \) T-ALL suggests a correlation between PJA-BP expression and commitment to the TCR \( \alpha \beta \) lineage.

We also performed EMSA experiments with nuclear extracts from cell samples of three patients with TCR \( \alpha \beta \) T-LGL and two patients with TCR \( \alpha \beta \) Sezary syndrome. No PJA-BP expression was found in these mature TCR \( \alpha \beta \) T cell malignancies. This further supports our hypothesis that PJA-BP expression is restricted to thymocytes, because these mature T cell malignancies are derived from mature peripheral T cells, whereas T-ALL are assumed to be derived from cortical thymocytes (3, 16).

We were not able to detect PJA-BP expression in precursor-B-ALL (\( n = 5 \)) or B-CLL (\( n = 5 \)) cell samples, but one patient with a precursor-B-ALL contained a very weak and vague retarded band of similar size as PJA-BP (data not shown). In contrast to our previous data using nuclear extracts from myeloid cell lines (6), no retarded band of similar size as the PJA-BP could be detected in five AML cell samples tested, representing different myeloid differentiation stages. Interestingly, a retarded band with only a slightly lower electrophoretic mobility compared to PJA-BP was observed in these AML cell samples (Fig. 3). Although we cannot fully exclude that this retarded band consists of a protein complex of PJA-BP with other proteins, we hypothesize that PJA-BP expression is specific for thymocytes of the TCR \( \alpha \beta \) lineage.

**PJA-BP expression and TCR \( \delta \) deletion in human thymocyte subpopulations**

Our EMSA experiments using human continuous T cell lines and T cell malignancies suggested that PJA-BP expression might be correlated with commitment to the TCR \( \alpha \beta \) lineage. Little is known about the lineage commitment of human thymocytes, although deletion of the TCR \( \delta \) gene (and in particular \( \delta \)Rec–\( \psi \)J\( \delta \) rearrangement) is thought to play an important role (3). Based on the very low frequency of \( \delta \)Rec–\( \psi \)J\( \delta \) rearrangements in human T cell lines and T cell malignancies, we recently hypothesized that these rearranged gene segments exist for only an extremely short period during thymocyte differentiation (5). This might explain the weak PJA-BP expression in our T-ALL samples, and the lack of correlation between PJA-BP expression and TCR \( \delta \) gene deletion in these cell samples. We therefore performed additional studies using freshly isolated human thymocytes to further investigate the correlation between PJA-BP expression and TCR \( \delta \) gene deletion.

We designed a hypothetical scheme for human T cell differentiation in the thymus, based on immunophenotyping (3, 17–24) (Fig. 4A). According to this scheme seven thymocyte subpopulations were isolated (Fig. 4A). DNA and nuclear extracts were isolated for Southern blot analysis with the \( \delta \)Rec probe TCRDRE and for EMSA experiments with the TCRAPJ-C1 probe respectively.

No preferential \( \delta \)Rec–\( \psi \)J\( \delta \) rearrangements could be detected by Southern blot analysis in the three most immature T cell subpopulations characterized by the phenotypes (Fig. 4B): CD34+ /CD1- /CD3- /CD4- /CD8- (fraction 1), CD34+ /CD1+ /CD3+ /CD4+ /CD8+ (fraction 2) and CD34+ /CD1+ /CD3- /CD4- /CD8+ (fraction 3). A weak \( \delta \)Rec–\( \psi \)J\( \delta \) rearranged band, derived from ~5% of the thymocytes, was detected in the thymocyte subpopulation obtained by depletion of CD3+ and CD8+ thymocytes (Fig. 4B; fraction 5). Based on these data we conclude that \( \delta \)Rec–\( \psi \)J\( \delta \) rearrangements start in the CD34+/CD1+ /CD3+ /CD4+ /CD8+ thymocyte differentiation stage. The next thymocyte subpopulation (fraction 6) was obtained by depletion for the activation markers CD69 and CD27 (24). This subpopulation, in which the majority of the cells (~80%) are double positive (CD4+/CD8+), showed a strong \( \delta \)Rec–\( \psi \)J\( \delta \) rearranged band (Fig. 4B), suggesting that the major part of the \( \delta \)Rec–\( \psi \)J\( \delta \) rearrangements occur in these double-positive thymocytes. Interestingly, our data therefore suggest that \( \delta \)Rec–\( \psi \)J\( \delta \) rearrangement occurs during or after thymocyte expansion (25). The \( \delta \)Rec–\( \psi \)J\( \delta \) rearranged band was also present in the CD69+/CD27+ subpopulation (fraction 7), which contains more mature (single-positive) thymocytes (24). We cannot determine whether this rearranged band represents new \( \delta \)Rec–\( \psi \)J\( \delta \) rearrangements or remaining rearrangements from earlier stages still present in genomic DNA or in circular excision products of ongoing \( \psi \)Rec–J\( \alpha \) rearrangements (26).

We also investigated the TCR \( \alpha \) gene status in the different
Fig. 4. TCR δ deletion and PJA-BP expression in human thymocyte subpopulations. (A) Hypothetical scheme for human T cell differentiation based on immunological marker analysis (3,17–24). The different isolated thymocyte subpopulations (fractions 1–7) are indicated with bars, directly below the scheme; the total thymocyte population is depicted as fraction 8. (B) Southern blot analysis of filters containing EcoRI-digested DNA hybridized with the δRec probe TCRDRE. (C) EMSA of DNA probe TCRApJ-C1 with crude nuclear extracts from thymocyte subpopulations. Numbering of the lanes in (B) and (C) is according to the subpopulations numbering outlined in (A). ND, not determined.
human thymocyte subpopulations. For this purpose we did not use Southern blot analysis because of the large number of different \( V_\alpha \) and \( J_\alpha \) gene segments. We therefore investigated the expression of TCR \( \alpha \) gene transcripts in the thymocyte subpopulations via Northern blot analysis (data not shown). TCR \( \alpha \) mRNA was absent in the immature thymocyte subpopulation depleted for CD3\(^+\) and CD8\(\beta\)\(^+\) cells (see fraction 4 of Fig. 4), whereas TCR \( \alpha \) mRNA was strongly expressed in the thymocyte subpopulation obtained by depletion of CD69\(^+\) and CD27\(^+\) cells (see fraction 6 of Fig. 4). These results are in agreement with previous studies of Ramiro et al. (27). Unfortunately we were not able to perform Northern blot analysis of the thymocyte subpopulation depleted for CD3\(^+\) and CD8\(\beta\)\(^+\) cells (fraction 5) because of shortness of RNA. Therefore, RT-PCR experiments were performed to detect \( V_\alpha - C_\alpha \) transcripts (see Methods) using RNA of the different subpopulations. As shown in Fig. 5, comparable to our Northern blot data, fraction 4 (CD3\(^-\) CD8\(\alpha\)-thymocytes) was negative for \( V_\alpha - C_\alpha \) transcripts, whereas fraction 6 (CD69\(^-\) CD27\(^-\) thymocytes) was strongly positive. We were able to detect low levels of \( V_\alpha - C_\alpha \) transcripts in fraction 5 (CD3\(^+\)CD8\(\beta\)) thymocytes after hybridization (Fig. 5). Our data seem to be in contrast with previous data of Ramiro et al. (27) who did not detect \( V_\alpha - C_\alpha \) transcripts before the TCR\(\beta\)^\(-\) CD4\(^+\)CD8\(^+\) cell stage. However, we found a variable expression of \( V_\alpha - C_\alpha \) transcripts in repeated experiments (data not shown) and used more PCR cycles then in Ramiro’s study, suggesting that only very low amounts of \( V_\alpha - C_\alpha \) transcripts are present in the thymocyte subpopulation depleted only for CD3\(^-\) and CD8\(\beta\)\(^-\) cells. Furthermore, we cannot formally exclude that these weak signals are derived from contaminating mature cells in this fraction of which >97% is CD3\(^-\)CD8\(\beta\). Our results show that \( \delta \)Rec\(\beta\)\(^-\) rearrangements start simultaneously with expression of TCR \( \alpha \) mRNA in thymocyte subpopulations. This further strengthens our hypothesis that \( \delta \)Rec\(\beta\)\(^-\) rearrangements exist for only an extremely short period during human thymocyte differentiation and are immediately followed by TCR \( \alpha \) rearrangements (5).

Our EMSA experiments using nuclear extracts from the thymocyte subpopulations revealed that PJA-BP is expressed in the immature CD3\(^+\) thymocyte subpopulation depleted for CD3\(^+\) and CD8\(\alpha\)\(^+\) cells (fraction 4), whereas low levels of PJA-BP expression could be detected in the thymocyte subpopulation depleted only for CD8\(\beta\)\(^+\) cells (fraction 5; Fig. 4C). The thymocyte subpopulation obtained by depletion of CD69\(^+\) and CD27\(^+\) cells (fraction 6; Fig. 4C) was strongly positive for \( V_\alpha \) transcripts (second panel) and \( V_\alpha - C_\alpha \) transcripts, whereas \( V_\alpha - C_\alpha \) transcripts in repeated experiments were very low. The primer combinations used specifically recognize \( V_\alpha \) and RNA from the B cell line RAJI was used as a negative control. Amplification of the ABL gene was used as a RNA control for the amount of RNA used in the PCR (fourth panel).

At first sight our results seem to be in contrast to the murine system, because several studies conclude that the \( \delta \)Rec\(\beta\)\(^-\) rearrangements do not play a significant role in murine T cell lineage commitment (26,29,30), whereas other studies indicate that also in mice the TCR \( \delta \) gene deletion process is an important phenomenon for the bifurcation of the TCR \( \alpha \beta/\gamma\delta \) lineages (31). These contradictory conclusions are mainly caused by the fact that multiple gene segments can mediate TCR \( \delta \) gene deletion in mice: in contrast to the single \( \delta \)Rec gene segment in man, at least three different murine \( \delta \)Rec gene segments exist that can use multiple J\(\beta\)’s as 3’ acceptor sites, including J\(\beta\)_9 (7,31). The usage of multiple TCR \( \delta \) deleting rearrangements in mice explains the relatively low frequency of \( \delta \)Rec\(\beta\)\(^-\) rearrangements in mice as compared to man (2). Nevertheless, several studies showed that rearrangements to the J\(\beta\)_9 gene segment occur at high frequency in immature murine thymocytes (26) as well as in early murine ontogeny (30). We therefore hypothesize that rearrangements to the J\(\beta\)_9 gene segment and the J\(\beta\)_9 binding protein PJA-BP play an important role in TCR \( \delta \) deletion and \( \alpha \beta \) lineage commitment in both man and mice.

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