Quantification of aminopeptidase N mRNA in T cells by competitive PCR

T. Wex[a,*], U. Lendeckel[a], H. Wex[b], K. Frank[a], S. Ansorge[a]

[a]*Institute of Experimental Internal Medicine, Department of Internal Medicine, University of Magdeburg, Leipziger Strasse 44, D-39120 Magdeburg, Germany*

[b]*Institute of Neurobiology, Department of Neurochemistry, D-39120 Magdeburg, Germany*

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Abstract The aminopeptidase N (CD13, EC 3.4.11.2) is a well-characterized surface molecule expressed in a variety of cell types and species. Recent data indicate an expression of the APN mRNA and the corresponding aminopeptidase activity in human peripheral T cells and related cell lines as well. Here, the sensitive method of competitive PCR was used to quantify low amounts of APN mRNA in T cell lines. An APN cDNA fragment enshortened by a deletion of 87 bp was used as an internal APN-specific standard. The myelo-monocytic cell line U937 and the lymphoid T cell lines HuT78 and H9 contain 2.3×10^7, 5.9×10^6 and 5.6×10^6 copies/μg total RNA, corresponding to 160, 70 and 50 copies/cell, respectively. These data have been confirmed by determination of the APN activity, that represents a fraction only of the total cellular neutral aminopeptidase activity in hematopoetic cells. In the case of the CD13-positive cell line U937, ~60–70% of the total neutral aminopeptidase activity could be attributed to APN. In contrast, only a minor fraction (5–20%) of the cellular neutral aminopeptidase activity in the T cell lines H9 and HuT78 represents APN. The results suggest that APN gene expression within the hematopoetic system is not restricted to myelo-monocytic cells, instead a low APN expression may be a common feature of lymphocytes, at least of T cells, too.

Key words: Aminopeptidase N; EC 3.4.11.2; T cell; Competitive PCR

1. Introduction

Aminopeptidase N (CD13, EC 3.4.11.2) is a metallopeptidase commonly expressed on the surface of various cell types. As far as cells of the hematopoetic system are concerned, aminopeptidase N was generally believed to be exclusively expressed on cells of the myelo-monocytic lineages. Aminopeptidase N has been well-characterized with respect to its cellular localization as well as its biochemical and structural features [1–4]. The enzyme is involved in the final hydrolysis of nutrients and degradation of bioactive molecules such as neuropeptides, angiotensin, tuftsin and cytokines [1,5–7]. Recent data indicate that aminopeptidase N serves as a receptor for different ligands [8–11] and that it is possibly involved in processes of antigen presentation [12].

Human lymphocytes and related cell lines are predominantly CD13-negative. There are several reports, however, describing the occurrence of neutral aminopeptidase activity on these cells [13–16,18]. These results suggest that the aminopeptidase N (CD13) is expressed in lymphoid cells too.

To address this assumption and to investigate aminopeptidase N-gene expression in lymphoid cells, we have created a competitive RT-PCR of APN mRNA. Furthermore, neutral aminopeptidase activity of T cell lines was characterized in more detail to answer the question whether their aminopeptidase activity could be attributed to APN.

2. Materials and methods

2.1. Cloning and partial deletion of an APN gene fragment

The 1865-bp *Eco*RI fragment of the APN cDNA (kindly provided by A.T. Look) was cloned into the plasmid pSPT19 (Boehringer Mannheim). The deletion of the APN cDNA fragment was achieved by BAL31 nuclease (USB) using the unique *Dra*III restriction site of the APN cDNA. Extent and position of the deletion were determined by sequencing using the 'cycle sequencing kit' (USB) and the DIG-labelled APN-specific primer A1. The sample was loaded on a 8% sequencing gel and electrophoretically separated by the direct blotting system GATC (MWG Biotec) as recommended by the manufacturer.

2.2. In vitro transcription

1 μg pSPT19/2–14 plasmid DNA was cut with *Bgl*I yielding three fragments. The 775-bp 'APN-like transcript' (Fig. 1) was synthesized by T7 polymerase using the 'Mega-script kit' (Ambion) following the manufacturer's instructions. Amount and integrity of the synthesized RNA were checked by denaturing agarose-gel electrophoresis. Dilutions of the standard RNA fragment were stored at −80°C.

2.3. Competitive PCR

2.3.1. Reverse transcription. RNA was extracted using the method of Chomczynski and Sacchi [20]. 50–300 ng total RNA from cells was mixed with an aliquot of a given dilution of the standard APN fragment on ice. Mixtures were transcribed into cDNA by 50 U moloney murine leukaemia virus reverse transcriptase as recommended by the manufacturer (USB) using 1 ng APN-specific primer A_{RT}.

2.3.2. Enzymatic amplification of the cDNA. 1 μl of the cDNA reaction mixture was used directly for enzymatic amplifications that were performed in 30 μl reaction volume containing 1 U *Taq* polymerase (Amersham), 5 mM dNTP, 0.2 pmol of primers A1 and A2 and 1× reaction buffer (Amersham) in an Autogene II (Grant). Initial denaturation at 94°C for 3 min was followed by 30 cycles with denaturation at 95°C for 0.7 min, annealing at 54°C for 0.6 min and elongation at 72°C for 1.3 min. The final extension step was at 72°C for 10 min. 5 μl of each reaction mixture was applied on an 1.8% agarose gel and stained with ethidium bromide. A photograph of the illuminated gel (Polaroid 667) was scanned using the densitometer CD60 (Desaga) at 410 nm.

2.3.3. Calculation of APN mRNA amounts. Peak areas of the densitograms correspond to fluorescence intensity of DNA bands. The ratio of the peak areas of the wildtype and standard fragments was calculated to determine APN mRNA amount. The APN mRNA copy numbers/μg total RNA have been calculated taking into account the molecular weight (M_r = 249077) of the 'APN-like transcript'. Absolute copy numbers of APN transcript/cell are based on the ascertained RNA contents of the cells.

*Corresponding author. Fax: (49) (391) 6713291.

Abbreviations: CD, cluster of differentiation; DIG, digoxigenin; RT, reverse transcription; PCR, polymerase chain reaction.

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2.4. Enzymatic assay

Total neutral aminopeptidase activity was measured by determination of the Ala-p-NA-hydrolysing activity of vital cells and corresponding cellular fractions, respectively. All measurements were made in triplicate as previously described [13]. The presence of APN was detected using APN-specific effectors, such as the anti-CD13 monoclonal antibody WM15 [21] and the APN-specific inhibitor probestin [22].

Briefly, cells were disintegrated by a 30-min incubation on ice in PBS 1% Triton X100, pH 7.4. Particulate material was removed by centrifugation at 4°C, 100.000 × *g* for 30 min. Intact vital cells and the corresponding lysates, respectively, were preincubated in the presence of the effectors (WM15; probestin, 10^{-6} M) at room temperature for 30 min. Afterwards, Ala-p-NA-hydrolysing activity was measured in the presence of the effectors [13].

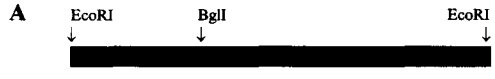
3. Results

3.1. Competitive RT-PCR

The clone lacking 87 bp of the APN cDNA fragment, named pSPT19/2–14, was chosen as the standard fragment for the competitive PCR (Fig. 1). 40 copies of the 'APN-like transcript' were detectable by RT-PCR (not shown). The same copy number has been determined after storage of the standard RNA at −80°C for 3 months. This implied that the standard RNA template is stable under these conditions (not shown). Titration experiments using wildtype and standard DNA fragments clearly showed linearity of the competitive system in a broad concentration range (Fig. 2). The linearity of the ratio wildtype/standard fragment is guaranteed over 6 consecutive dilution steps (dilution factor 2) if the ratio of both fragments amounts among 0.4 and 16. The ratio of the wildtype/standard fragment of a given sample was identical after 25, 30 and 35 cycles, respectively (not shown).

3.2. Quantification of APN mRNA content of cell lines

Quantification of the APN mRNA content in cell lines was performed in two steps as described above. The cell line U937



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+1   ATGgccaagg gcttctatat ttccaagtcc ctgggcatcc tggggatcct gggcgtggca
          ──────── A1 ────────→
+61  gccgtgtgca caatcatcgc actgtcagtg gtgtactccc aggagaagaa caagaacgcc
+121 aacagctccc ccgtggcctc caccacccag tccgcctcag ccaccaccaa ccccgcctcg
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              ←──────── A2 ────────
+481 ctggtggtgc acctcaaggg ctccctggtg aaggacagcc agtatgagat ggacagcgag
                          ←──────── ART ────────
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+601 aatgtcagaa aggtggtggc cactacacag atgcaggctg cagatgcccg gaagtccttc
+661 ccatgcttcg atgagccggc
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Fig. 1. Partial map and sequence of the APN cDNA fragment, cloned in the plasmid pSPT19/2–14. Part A gives locations of the deletion in the clone pSPT19/2–14 and of *BglI* site, used for restriction of plasmid DNA prior the in vitro transcription. B shows a part of the APN cDNA, sequence lacking in the clone pSPT19/2–14 (capital and bold letters) and the primer binding sites of primers A1, A2 and A_{RT}, those were used in RT-PCR. The sequence starts with the first codon (ATG) and is presented as far as the *BglI* restriction site. The 775-bp 'APN-like transcript' consists of a 64-bp vector fragment, the 118-bp region that is located downstream of the start codon and the given cDNA sequence (593 bp). Complete APN cDNA sequence was published by A.T. Look and co-workers [4].

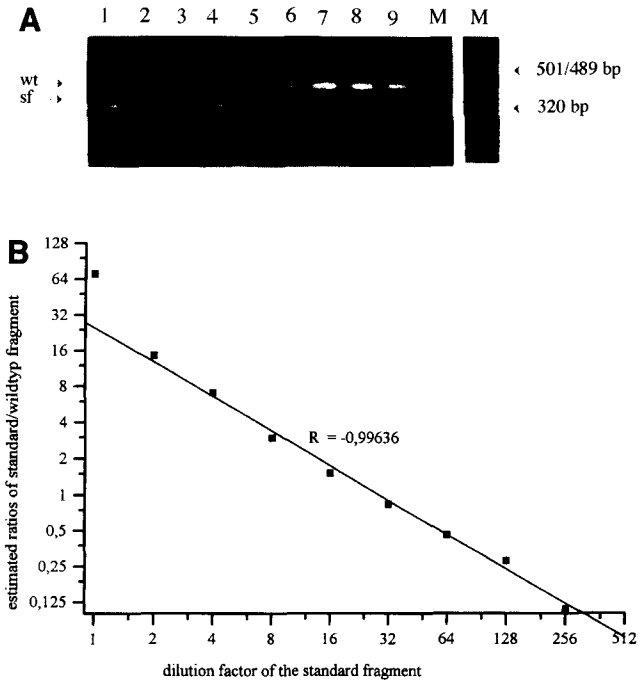

Fig. 2. Usage of the APN standard fragment in the competitive PCR. (A) PCR on dilutions of DNA mixes, consisting of wildtype (wt) and standard DNA (sf). 0.3 ng wildtype plasmid-DNA (pSPT19/2) was mixed with decreasing amounts of standard plasmid-DNA (pSPT19/2–14; lane 1–9: 10 ng–39 pg; dilution factor 2) and amplified as described. The sizes of both fragments are 468 bp for APN cDNA fragment and 381 bp for the standard fragment, respectively. M, molecular weight marker VIII (Boehringer Mannheim) (B) Plot of determined ratios from consecutive dilutions of wildtype and standard fragment. Ratios were obtained by scanning photograph given in Fig. 2A. Dilution factors (1–256) correspond to lanes 1–9, illustrated in Fig. 2A. The equimolar concentration of both fragments was calculated at dilution factor 28. The determined ratios were fitted among dilution factors 2 and 128. These results were confirmed by three additional experiments (not shown).

(Fig. 3) as well as the two T cell lines H9 and HuT78 (not shown) contain APN mRNA. 2.3×10^7, 5.9×10^6 and 5.6×10^6 copies/μg total RNA, respectively, were calculated for the myelo-monocytic cell line U937 and the lymphoid T cell lines HuT78 and H9. Taking into account the RNA content, U937 holds ~160 copies of APN mRNA/cell whereas the T cell lines H9 and HuT78 contain 50 and 70 copies, respectively (Table 1).

3.3. Detection of APN-derived enzymatic activity

In contrast to the myelo-monocytic cell line U937, T cell lines H9 and HuT78 are CD13-negative using immunfluorescense analysis (not shown). In spite of this fact, both T cell lines express considerable neutral aminopeptidase activities, measured by the cleavage of the chromogenic substrate Ala-p-NA (Table 1). However, only the aminopeptidase activity of U937 cells could be significantly inhibited by the anti-CD13 monoclonal antibody WM15. In contrast, aminopeptidase activities of both T cell lines were not affected (Table 1). Furthermore, the inhibitor probestin (10^{-6} M) strongly affects aminopeptidase activity of vital U937 cells (18% residual activity) but the corre-

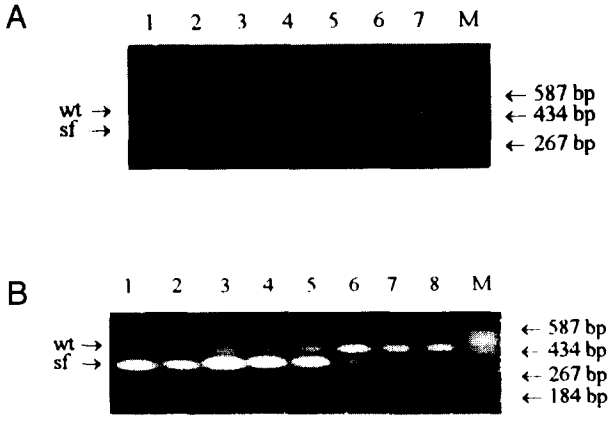

Fig. 3. Determination of the APN mRNA content of the myelomonocytic cell line U937. (A) 500 ng total RNA from U937 was mixed with the APN standard fragment (4×10^8–10^2 copies), reverse-transcribed and amplified using primers A1 and A2 (lanes 1–7). (B) 500 ng total RNA from U937 was mixed with the APN standard fragment (2.6×10^8–2×10^6 copies), reverse-transcribed and amplified as described above (lanes 1–8). M, molecular weight marker V (Boehringer Mannheim).

sponding activities of H9 and HuT78 cells were decreased to 58 and 78%, respectively, only (not shown).

4. Discussion

Experiments reported in this paper are aimed at the detection of APN transcript using the competititive RT-PCR technique. APN-specific mRNA is detectable and could be quantified in the lymphoid cell lines HuT78, H9 and in the CD13-positive cell line U937, respectively. The usage of intron-overspanning primers in the experiments proved that amplified DNA fragments indeed originate from APN mRNA, instead of from contaminating by DNA. Data obtained from competition of the APN standard by the APN wildtype fragment clearly demonstrate that this RT-PCR system works well over a broad range. The experimental determined ratios of the fluorescence intensities of the DNA fragments (wildtype and standard) accurately correspond to the given dilution factor (Fig. 2A,B). Therefore, it could be concluded that absolute values of these ratios among 0.4 and 16 are suitable for exact quantification of APN mRNA transcript level, and it is not necessary to titre the amount of standard RNA to equal amounts of the APN mRNA to ensure a ratio of nearly 1.

Data presented here provide evidence that the APN gene is really expressed in T cells. Furthermore, the results are in agreement with qualitative RT-PCR analysis on peripheral T cells and T cell lines [13,17]. The detection of APN transcripts in T cells contradicts to previous studies reporting that the APN expression is restricted to the myelo-monocytic cells and their progenitors [1–4,19]. The attempts of others to detect APN mRNA in lymphoid cells by Northern blotting failed until yet, suggesting a low copy number of APN gene transcript in these cells [19]. The detected copy numbers in a range of 50–160 copies/cells confirm this assumption. The values are in the range of class II mRNAs, occurring at 20–3500 copies/cell. The discrepancy between the Northern analysis and the RT-PCR may be explained by the higher sensitivity of the PCR method.

The CD13-positive U937 contains 2–3 times more APN transcript/cell than both T cell lines found to be CD13-negative. The APN copy numbers of the cells, determined by the RT-PCR, have been confirmed by analysis of the APN-derived enzymatic activity. Although H9 and HuT78 cells express neutral aminopeptidase activities equal to or higher than U937, respectively, only a minor fraction of the aminopeptidase activity in T cells really originates from the APN gene. This could be concluded from the lack of inhibition of the enzymatic activity by the antibody WM15, strongly decreasing APN activity [21] and the low inhibition of enzymatic activity by the APN-specific inhibitor probestin [22]. Summarizing these data, it could be concluded that only 5–20% of total neutral aminopeptidase activities in T cells represent membrane-bound APN. In contrast, 60–70% of the neutral aminopeptidase activity are derived from the APN gene in U937 cells. Furthermore, the aminopeptidases from U937 cells and T cells (H9 and HuT78) differ in their molecular weights, isoelectric points, inhibition rates by inhibitors and cellular localizations (unpubl. data).

APN mRNA copy numbers determined in our experiments are specific with respect to the proliferative status of the cells. Changes of the RNA content, normally occurring during different growth phases of cells, strongly affect cellular copy numbers of transcripts. To circumvent these variations, cells were harvested in each experiment at the beginning of the stationary phase after 72 h.

Results reported in the present study show that APN gene expression is not restricted to myelo-monocytic cells, clearly T cells and derived cell lines are able to express aminopeptidase N although on a low level. The main aminopeptidase expressed in T cells seems to be a previously unknown enzyme, which is

Table 1
APN mRNA contents and Ala-p-NA-hydrolysing activity of myelo-monocytic cell line U937 and T cell-derived cell lines H9 and HuT78

Cell line	APN mRNA copies/μg total RNA	Total RNA μg/10^6 cells	APN mRNA copies/cell	Ala-p-NA hydrolysis (pkat/10^6 cells)	Inhibition of Ala-p-NA hydrolysis by WM15 (residual activity, % of control)
U937	2.3×10^7	7	160	84.8 ± 3.2	58.4 ± 13.5
HuT78	5.9×10^6	9	50	108.2 ± 22.2	99.8 ± 0.5
H9	5.6×10^6	12	70	66.1 ± 14.8	98 ± 3.2

All values are at least means of two experiments and correspond to cells harvested in the early-stationary phase. Enzymatic activity and its inhibition by the anti-CD13 monoclonal antibody was measured in the lysate of cells.

not related to APN, and which has to be characterized in more detail.

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