The activation-dependent induction of APN-(CD13) in T-cells is controlled at different levels of gene expression

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Abstract Recently, it was shown that aminopeptidase N (E.C. 3.4.11.2, CD13) is up-regulated during mitogenic stimulation of peripheral T-cells. In this study, we demonstrate that the half-life of APN mRNA was considerably prolonged in these cells leading to a 2.7-fold increase of APN transcript level. The apparent half-life time of the APN transcript was investigated by the RNA synthesis inhibitor-chase method using actinomycin D. The steady-state APN mRNA levels was determined by a competitive RT-PCR. The half-lives estimated in resting T-cells, natural killer cells and permanently growing tumour cells varied between 3.5 and 6 h. Finally, nuclear run-on assays revealed that the *APN* gene expression of stimulated T-cells is controlled by increased promoter activity as well. These studies suggest a control of *APN* gene expression at the post-transcriptional level in addition to promoter-mediated regulation.

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Key words: CD13; E.C. 3.4.11.2; Actinomycin D; Half-life time

1. Introduction

The cell-surface antigen CD13, also known as aminopeptidase N (APN, E.C. 3.4.11.2), is a 150 kDa glycoprotein [1,2]. It is a stalked integral membrane peptidase, mainly expressed in the small intestinal and kidney brush borders but also found in brain, lung, liver and in the hematopoietic system [3–5]. Studies aimed at the functional role of APN revealed that this enzyme is involved in different processes such as hydrolysis of nutrients, inactivation of bioactive peptides, binding of corona viruses, mediating CMV infection, regulation of tumour-cell invasion, and degradation of the extracellular matrix and antigen presentation [3,6–12].

Until recently, in the hematopoietic system, CD13 expression was exclusively detected on myelomonocytic cells [2,5]. However, it is now well established that increased CD13 surface expression is frequently detected on malignant B-cells, tumour-infiltrating lymphocytes (TIL), T-lymphocytes derived from inflamed tissues as well as mitogen activated T-cells [13–16]. The induction of CD13 expression on mitogen-activated T-cells as well as increased CD13 surface expression on T-cells derived from inflamed tissues is always accompanied by in-

creased levels of corresponding enzymatic activity and APN mRNA [13,16,19].

The aim of this study was to investigate *APN* gene regulation in mitogen-stimulated T-cells. Here, we present data suggesting that *APN* gene expression of these cells is controlled at both the transcriptional and post-transcriptional level.

2. Materials and methods

2.1. Cell lines, culture, preparation and mitogenic stimulation of cells

U937 and H9 cells were obtained from ATCC (USA) or DSM (Braunschweig, Germany), respectively. Cells were grown in IMDM (Gibco) supplemented with 10% foetal calf serum (Gibco BRL) and 60 U/ml penicillin (Gibco BRL).

Peripheral T-cells were enriched from the MNC population by nylon adherence as described by others [17]. FACS analyses indicated a purity of CD3$^+$ cells between 85 and 95%. Natural killer (NK) cells were enriched by depletion of CD3$^+$ and CD19$^+$ cells using the MACS system (Miltenyi Biotec) as previously described [18]. In general, NK cells consist of more than 90% CD16$^+$/CD56$^+$ cells, whereas CD3$^+$ cells were less than 5%.

Purified T-cells were stimulated by 5 µg/ml PHA (Boehringer Mannheim) in the presence of 10 ng/ml PMA (Sigma) for 3 days [16]. NK cells were stimulated by 1000 U/ml IL-2 for 3 days [18].

2.2. Enzymatic assay

Ala-pNA hydrolysing activity of cells or their lysates, respectively, was determined in triplicate by measuring the hydrolysis of the chromogenic substrate Ala-pNA (2.5 mM) as described previously [16].

2.3. Competitive PCR

RNA samples of resting or stimulated cells were obtained using the 'RNeasy' kit (Qiagen) according to the manufacturer's instructions. Total RNA (250 ng-1 µg) was mixed with an aliquot of a standard APN mRNA fragment and transcribed into cDNA by 50 U of MMLV reverse transcriptase (USB) using an APN-specific primer (5′-gcggtagaagcccgccaggtca).

An aliquot of the cDNA mixture was used directly for enzymatic amplifications which were performed in a 40 µl reaction volume containing 0.5 U 'Gold-Star' polymerase (Eurogentec), 0.25 mM dNTP, 2.5 mM MgCl$_2$, 0.2 pmol of primers (forward: 5′-gccgtgtgcacaat-catcgcact; reverse: 5′-caccagggagcccttgaggtg) and 1× reaction buffer (Eurogentec) in the thermocycler Autogene II (CLF, Emmersacker, Germany). Initial denaturation at 94°C for 3 min was followed by 30 cycles with denaturation at 96.5°C for 0.4 min, annealing at 54°C for 0.6 min and elongation at 72°C for 0.8 min. The final extension step was 72°C for 10 min. An aliquot of the PCR reaction was electrophoretically separated in a 1.6% agarose gel (Biozym, Germany), documented by a video image system (GelPrint 2000i, MWG Biotech, Germany) and densitometrically analysed using RFLP 3.0 software (Scanalytics, USA).

2.4. Nuclear run-on assay

Nuclear run-on assays were performed from isolated nuclei derived from resting and stimulated T-cells as described by others with some modifications [20]. Briefly, 1–2×10^7 cells were harvested, incubated on ice for 10 min, washed twice in ice-cold PBS (pH 7.4) and lysed in NP-40 buffer (10 mM Tris-HCl, pH 7.4; 10 mM NaCl; 3 mM MgCl$_2$; 0.5% NP-40). After an incubation on ice for 5 min, nuclei were isolated by centrifugation (500×g, 4°C, 5 min), resuspended in NP-40

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Abbreviations: APN, aminopeptidase N; Ala-pNA, alanine-*p*-nitroanilid; CD, cluster of differentation; EDTA, ethylenediaminetetraacetic acid; cpm, counts per minute; mab, monoclonal antibody; PBS, phosphate-buffered saline; PHA, phytohemagglutinine; PMA, phorbol 12-acetate 13-myristate; RT-PCR, reverse transcription-polymerase chain reaction; SDS, sodium dodecyl sulfate; SSC, standard sodium citrate
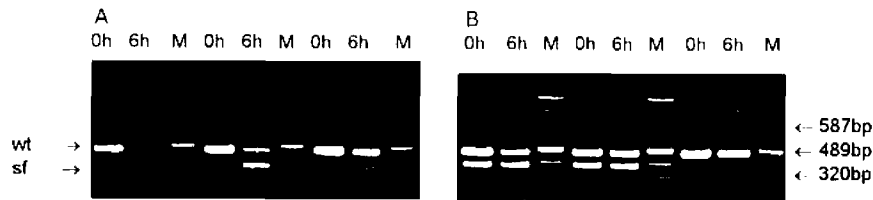
Fig. 1. Determination of the APN mRNA content of restin (A) or stimulated (B) T-cells after RNA synthesis inhibition by actinomycin D. Cells were cultured in the presence of 5 µg/ml actinomycin D for 6 h, then their APN-transcript levels were estimated as described in Section 2. The figure represents three independent experiments using the same T-cells. Equal amounts of RNA and standard fragment were used in the both samples of each experiment (0 h, 6 h). Summarizing data from six T-cell populations, resting or stimulated T-cells contained 2.9 ± 1.6 or 7.8 ± 3.9 pg APN mRNA/µg RNA, respectively.

buffer again and centrifugation was repeated. The nuclear pellets were resuspended in 100 µl of storage buffer containing 50 mM Tris-HCl (pH 8.3), 40% glycerol, 5 mM MgCl₂, 0.1 mM EDTA and stored at −70°C until use.

The frozen nuclei (100 µl) were mixed with 100 µl of 2× reaction buffer (10 mM Tris–HCl, pH 8.0; 5 mM MgCl₂; 0.3 M KCl; 5 mM DTT; 1 mM ATP; 1 mM CTP; 1 mM GTP; 50 µM UTP) (Boehringer Mannheim) and 10 µCi [α³³P]UTP (800 Ci/mmol, ICN Biomedicals, Germany). The reaction mixture was incubated at 30°C for 30 min. The labelled nuclear extract was subsequently treated with DNase I, proteinase K and then twice extracted with phenol/chloroform, precipitated by ethanol, dried and resolved in 200 µl of aqua bidest.

An aliquot of this solution was used to determine the specific activity of the nuclear lysate using the counter SMG 20046 (RFT, Germany). DNA slot-blots were prepared using the plasmid pSKAPN containing the full-length APN cDNA (kind gift from A.T. Look [4]).

Hybridization experiments were performed in 5×SSC containing 2% blocking reagent (Boehringer, Mannheim), 0.1% N-laurylsarcosine, 0.02% SDS, 50% formamide at 42°C for 20 h. Membranes

were washed twice in 2×SSC at room temperature for 15 min and once in 1×SSC at 42°C for 30 min. Hybridization signals were detected using a phosphoimager (Bas 1000, Fuji) and quantified using software RFLP 3.0 (Scanalytics, USA).

3. Results

3.1. Half-life time of APN transcripts in hematopoietic cells

The rate of decay of APN mRNA in different hematopoietic cells was investigated using the RNA synthesis inhibitor actinomycin D. Cells were cultured in the presence of 5 µg/ml actinomycin D for 1–6 h. At different times, their APN mRNA content was quantified using a competitive RT-PCR approach. The inhibition of newly transcribed messenger RNA by actinomycin D leads to a continued decrease of mRNA species, the time course of which is depended on their rates of decay. The reduction of APN RNA level is
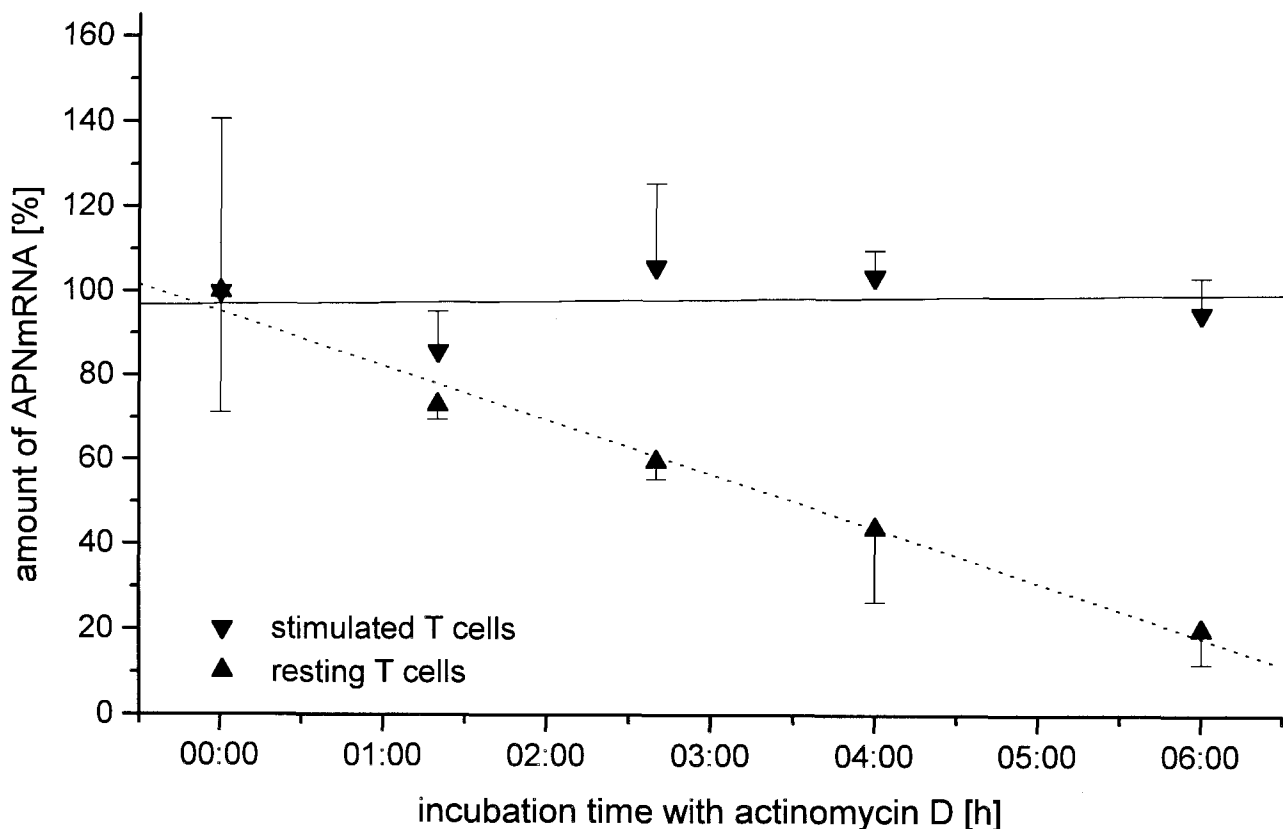

Fig. 2. Estimation of APN mRNA half-life time in resting and mitogen-stimulated T-cells. The graphs represent data obtained by competitive RT-PCR as shown in Fig. 1 using T-cells derived from six healthy donors.

Table 1
Half-life times of APN transcripts in different hematopoietic cells

Cells	Half-life of APN mRNA(h)	APN mRNA stabilization after stimulation
Resting T-cells	3.5 ± 0.7 (n = 6)	yes
Resting NK cells	6 ± 1.9 (n = 3)	yes
T-cell line H9	3.5 ± 1.9 (n = 3)	yes
Histiocytic cell line U937	4.5 ± 0.9 (n = 3)	not determined

Half-life times were determined by competitive RT-PCR as demonstrated in Fig. 1.
Data represent means of at least three experiments (n).
No noticeable decrease of steady-state APN mRNA level was observed in stimulated T-, NK and H9 cells until 6 h co-culture with actinomycin D as shown for stimulated T-cells in Fig. 2.

measured as the time-dependent decrease of the ratio between the APN wild-type and the APN standard fragment in RT-PCR. Fig. 1A represents three independent quantification experiments using resting T-cells from one donor. Summarizing the data obtained with resting T-cells from six healthy donors, the half-life time of APN transcript in resting T-cells was determined to be approximately 3.5 h (Fig. 2). The apparent half-life of APN transcript in resting NK cells was estimated as about 6 hours, however, NK-cell data showed more variance than those of T-cells (Table 1). The APN mRNA half-lives of permanently growing tumour cells such as the T-cell line H9 or the histiocytic cell line U937 were estimated to be 3.5 or 4.5 h, respectively (Table 1).

3.2. Stabilization of APN transcript and increase of APN gene promoter activity in stimulated T-cells

Purified resting T-cells were stimulated for 3 days as described in Section 2. The induction of APN expression was confirmed by measuring APN-derived enzymatic activity. For instance, stimulated T-cells contained 15–30 pkat/10^6 cells Ala-pNA hydrolysing activity compared to 4–7 pkat/10^6 cells determined in resting T-cells. The steady-state level of APN mRNA was increased 2.7 ± 1.4-fold in these cells. Interestingly, the half-lives of the APN transcript was considerably prolonged in stimulated cells compared to their unstimulated counterparts. The APN mRNA level of stimulated T-cells remained unchanged up to 6 h of cultivation in the presence of actinomycin D as shown in Figs. 1B and 2. Furthermore, a stabilization of APN transcript was also observed in stimulated NK and H9 cells (data not shown).

To investigate whether the promoter activity of the APN gene additionally contributes to the increased steady-state APN transcript levels in stimulated T-cells, nuclear run-on assays using nuclei derived from these cells were performed. Slot-blots were hybridized with equal amounts of labelled nuclear lysates. Densitometrical analysis of hybridization signals revealed an increase of promoter activity up to 172.5 ± 38.9% (n = 3) in stimulated T-cells compared to those of resting T-cells (data not shown).

4. Discussion

During the last years it has been shown that within the hematopoietic system CD13 expression is not restricted to myelomonocytic cells. It has been longly known that this antigen is frequently expressed on lymphoid tumour clones. The frequencies of such CD13-positive clones vary between 5 and 30% [13,21]. Recently, it has been proven that other immune cells such as resting lymphocytes and NK cells express membrane-bound APN as well. In contrast to myelomonocytic

cells, these cells express low amounts of APN; however, their CD13 surface expression is increased by different mitogenic stimuli or after co-cultivation with synoviocytes or other CD13-expressing cells [16,22–24]. The functional relevance of this induction is unclear as yet, but it has been proposed that APN like other membrane-bound peptidases such as neutral endopeptidase (E.C. 3.4.24.11, CD10), aminopeptidase A (E.C. 3.4.11.7) or angiotensin-converting enzyme (E.C. 3.4.15.1, CD143) may function as a regulatory molecule that degrades bioactive peptides [5]. The induction of CD13 on T-cells is generally accompanied by an increase of the corresponding enzymatic activity and APN mRNA level [16,24]. To gain insight into the mechanism underlying the CD13 induction on T-lymphocytes, we investigated the transcriptional regulation of the APN gene. Actinomycin D inhibits nearly all RNA synthesis by intercalation into DNA. Furthermore, it also prevents the binding of ribosomes to transcripts and, therefore, subsequent protein synthesis [25]. Using this approach, the half-lives of the APN transcript were determined in different immune cells. The average half-life span of human transcripts is about 10 h, ranging from less than 15 min to more than 24 h [26]. Talking into account these data, the APN half-lives of 3.5–6 h could be considered as medium. Since no data on APN half-life time in any tissue are available, the data obtained in this study can only be compared to those of other glycoproteins. Comparable half-life times were described for annexin VII (3–6 h), lactoferrin (8–9 h) and myeloperoxidase (4–5 h) [27,28].

The transcriptional regulation of APN gene expression was revealed by two excellent studies. Both Shapiro and co-workers as well as Olsen and co-workers identified separate promoters that initiate transcription in intestinal epithelial and myeloid cells. In epithelial cells, transcription is controlled by a classical promoter containing a TATA box immediately upstream to the first codon of APN, whereas an alternative GC-rich promoter located about 8 kb upstream from coding region is active in myeloid cells as well as in fibroblasts [29,30]. Both promoter regions considerably differ with respect to transcription factor-binding sites. The transcription factors LF-B1 and Sp-1 represent the major regulators in epithelial and liver cells, whereas myb and Ets-2 both transactivate CD13 expression in myeloid cells [29,31]. The importance of promoter-mediated regulation is emphasised by reports showing that modulation of APN surface expression on endometrial cells and leukaemic B-cells is directly linked to the activity of the tissue-specific APN gene promoter [32,33].

The transcription of the APN gene in hematopoietic cells is controlled by the alternative promoter [31]. RNA synthesis inhibition leads to a linear decrease of steady-state APN mRNA levels with half-life times of 3.5–6 h in different hem-

atopoietic cells. Therefore, it could be assumed that factors involved in APN mRNA degradation seem to be more stable than transcription factors which control the constitutive *APN* gene expression in resting T-cells. The activation-dependent induction of *APN* gene expression in T-cells may be due to the action of new transcription factors, an assumption which is supported by the increase of APN-promoter activity detected in stimulated T-cells. Shapiro and co-workers identified putative Sp-1, PU- AP-2, myb- and ETF-binding sites in the alternative promoter which probably contributes to the transcriptional activation of the *APN* gene [30]. However, the prolonged half-life time of APN-transcript in stimulated T-cells as well as the discrepancy between the 2.7-fold increase of APN transcript level and the 1.7-fold rise of transcription rate in these cells suggest the appearance or disappearance of factors directly involved in the degradation of APN transcript. Post-transcriptional regulation of mRNA decay after stimulation is a common regulatory mechanism described for other transcripts such as the CD11a, the mMCP-2 (chymase-homolog protein) [34,35].

Taken together, the results of this study clearly show that the *APN* gene expression of stimulated T-cells is controlled at both the transcriptional and post-transcriptional level. Since stabilization of APN mRNA was observed in other stimulated cells such as H9 and NK cells, as well, it may be concluded that this process represents a general mechanism of APN induction in hematopoietic cells.

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