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Inhibition of Alanyl-Aminopeptidase Suppresses the Activation-Dependent Induction of Glycogen Synthase Kinase-3β (GSK-3β) in Human T Cells

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Inhibition of alanyl-aminopeptidase (APN, CD13) gene expression or enzymatic activity compromises T cell proliferation and function. Molecular mechanisms mediating these effects are not known as yet. Recently, we found the expression of the proto-oncogen Wnt-5a to be strongly affected by APN-inhibition. Wnt-5a and other members of the Wnt family of secreted factors are implicated in cell growth and differentiation. Here, we analyzed by quantitative RT-PCR and immunoblotting the expression in mitogen-activated T cells of a major constituent of the Wnt-5a pathway, glycogen synthase kinase-3β (GSK-3β). T cell activation by phytohaemagglutinin or pokeweed mitogen results in a strong increase of GSK-3β mRNA amounts. At the protein level, we observed an up-regulation of both GSK-3β and phosphorylated GSK-3β. This induction-dependent increase of GSK-3β is markedly reduced in response to inhibitors of alanyl-aminopeptidase, actinonin, leuhistin, and RB3014. These findings may provide a rational for the growth inhibition resulting from a diminished expression or activity of alanyl aminopeptidase. © 2000 Academic Press

Key Words: **alanyl aminopeptidase; CD13; cell proliferation; Wnt-5a; GSK-3β; quantitative PCR; Lightcycler.**

Alanyl aminopeptidase (EC 3.4.11.2, APN, CD13) is an 150 kDa metalloprotease of the M1 family of peptidases (clan MA, gluzincins; [1]) which is expressed as a homodimer on the surface of various cells. The human APN gene was cloned and mapped to chromosome 15 (q25-q26) [2, 3]. Its coding sequence is spread over 20 exons [4]. Among leukocytes APN is predominantly expressed on myelo-monocytic lineage cells [5]. Malignant transformation, inflammation or T cell activation may induce APN gene and surface expression on B and T cells [6–10]. Mutations in the APN gene may well contribute in the malignant transformation of leukocytes [11].

APN is involved in the degradation of neuropeptides and cytokines [12] [13–15] and may contribute in extracellular matrix degradation [16] (for review see [17]). Moreover, APN functions as a receptor for corona virus [18–20] and CMV [21] and has been suggested to contribute in antigen processing [22].

Antisense-mediated inhibition of alanyl aminopeptidase gene expression as well as inhibition of its enzymatic activity have been shown previously to compromise proliferation and DNA-synthesis of peripheral T cells [6, 7, 23, 24], of the T cell line H9, and of the promyeloic cell line U937 [25]. Recent studies identified MAP kinases Erk1/Erk2 [26] and the proto-oncogen Wnt-5a [27] as molecular players possibly mediating the inhibitory effects on T cell growth and function of the aminopeptidase inhibitors, probestin and actinonin. Members of the highly conserved Wnt family of glycosylated and secreted factors regulate cell–cell interactions during developmental decisions through activation of receptor-mediated signalling pathways [28]. Wnt proteins upon binding to the seven-pass transmembrane receptor frizzled (Fz) transduce their signals through dishevelled (dsh) proteins to inhibit glycogen synthase kinase-3β (GSK-3β). Consequently, β-catenin accumulates in the cytosol leading to the activation of TCF/LEF-1 transcription factors (for review see: [29]). Furthermore, Wnt-5a is a hematopoietic growth factor [30] and may function as a tumor suppressor [31, 32].

The aim of this study was to analyze in activated human T cells the effect of alanyl-aminopeptidase inhibitors on the expression and activity of GSK-3β, an inherent component of the Wnt-5a signalling pathway.

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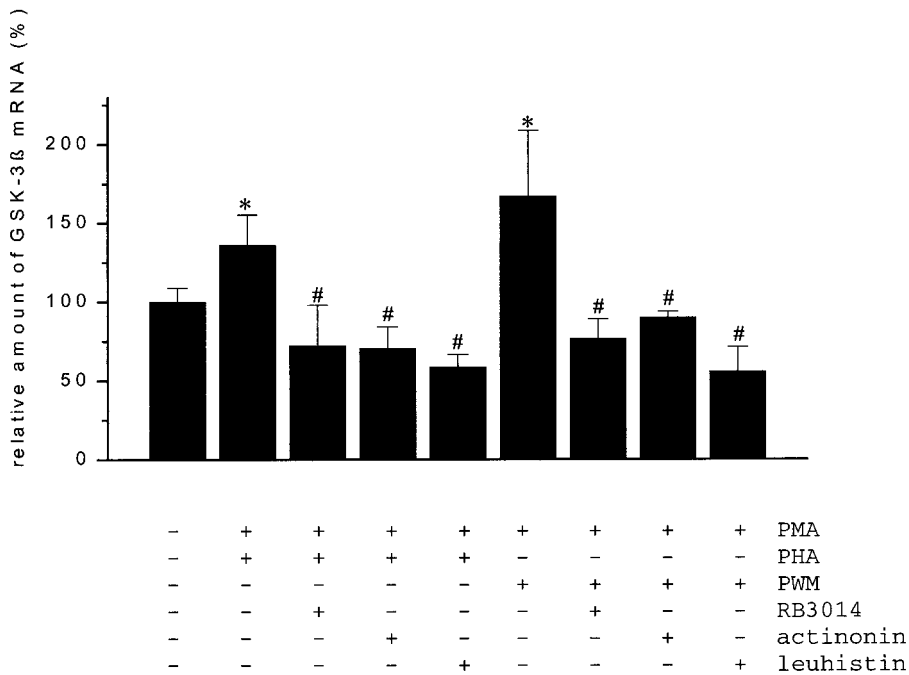

FIG. 1. Quantitative determination of GSK-3β mRNA content in resting (control) and activated human T cells. T cells were stimulated by either 1 μg/μl phytohemagglutinin (PHA) or 1 μg/μl pokeweed mitogen (PWM) together with 10 nmol/l phorbol-12-myristat-13-acetate (PMA) for 24 h. In the experiments indicated 10 μmol/l of actinonin, 10 μmol/l leuhistin, or 100 nmol/l RB3014 were added to the culture medium simultaneously with the T cell stimulants. After 24 h cells were harvested and relative GSK-3β mRNA content was analysed by quantitative PCR using the Lightcycler LC24. (*$P < 0.01$, compared to control; #$P < 0.01$, compared to PHA/PMA or PWM/PMA; $n = 4$).

MATERIALS AND METHODS

Reagents. Actinonin (3-[[1-[[2-(hydroxymethyl)-1-pyrrolidinyl]-carbonyl]-2-methyl-propyl]carbamoyl] octanohydroxamic acid [33] and leuhistin (3-amino-2-hydroxy-2-[1H-imidazol-4ylmethyl]-5-[methyl-hexanoic acid) [34] were purchased from Sigma. Anti-GSK-3 was purchased from Upstate Biotechnology and anti-phosphoSer9-GSK-3β was from New England Biolabs. RB3014, a very potent and selective inhibitor of APN, was designed and synthesized by Chen, Roques and Fournié-Zaluski as described recently [35].

Cell culture. Mononuclear cells (MNC) were prepared from peripheral blood of healthy donors by Ficoll-Paque gradient centrifugation [36]. T cells were enriched from the MNC fraction by the nylon wool adherence technique [37]. T cells were kept overnight in IMDM-medium and then cultured at a density of 1×10^5 cells/ml with the additions and for periods of times indicated in the figures.

RNA-isolation and quantitative RT-PCR. Total RNA was prepared by means of the RNeasy kit (Qiagen). 2 μg RNA were reverse-transcribed using AMV-RT (Stratagene) and 1/10th of the cDNA was used for RT-PCR.

Quantitative determination of GSK-3β mRNA contents was performed using the Lightcycler LC24 (Idaho Technology). A 10 μl reaction mixture consisted of 1 $\times$ reaction buffer with BSA (Idaho Technology), 3 mM $MgCl_2$, 200 μmol dNTP, 0.4 U InViTaq (InViTek), 0.2 μl of a 1:1000 dilution of SYBR-Green I (Molecular Probes), and 0.5 μmol of the gene specific primers GSK-3β-US (5′-GAAGAGAG-TGATCATGTCAG) and GSK-3β-DS (5′-CTTTCTTCTCACCACTG-GAG). Initial denaturation at 95°C for 10 s was followed by 40 cycles with denaturation at 95°C for 0 s, annealing at 62°C for 3 s, and elongation at 72°C for 12 s. The fluorescence intensity of the double-strand specific SYRB-Green I, reflecting the amount of formed PCR-product, was read at the end of each elongation step. 18 S mRNA amounts were determined using the RT primer pair commercially available from Ambion and used to normalise sample cDNA content.

Immunoblotting. Cells were resuspended in 20 mM HEPES buffer plus 1 volume 4 $\times$ RotiLoad (Roth). Aliquotes of equal protein content were separated on 4–20% gradient NuPage gels (Novex) using MES–SDS running buffer. After blotting on BAS-85 membrane (Schleicher & Schüll) GSK-3β protein was detected by means of anti-GSK-3 mab (Upstate Biotechnology) or anti-phospho-Ser9-GSK-3β mab (New England Biolabs) and goat-anti-mouse-POD/ Super Signal Extended Duration chemoluminescence substrate (Pierce). GSK-3β and phospho-GSK-3β were quantified by densitometric analysis using the RFLP-Scan software (Scanalytics).

RESULTS

Quantitative PCR

Using the Lightcycler LC24 we observed an activation-dependent increase of GSK-3β mRNA amounts in human T cells. This induction of GSK-3β mRNA was consistently seen, irrespective of T cells being stimulated by PHA/PMA or PWM/PMA. T cell activation in the presence of the inhibitors of alanyl-aminopeptidase, actinonin, leuhistin or RB3014, resulted in a significant suppression of GSK-3β mRNA induction (Fig. 1).

Both the activation-dependent induction of GSK-3β mRNA and its suppression by aminopeptidase inhibitors were detectable already one day after stimulation and lasted at least until day 3.

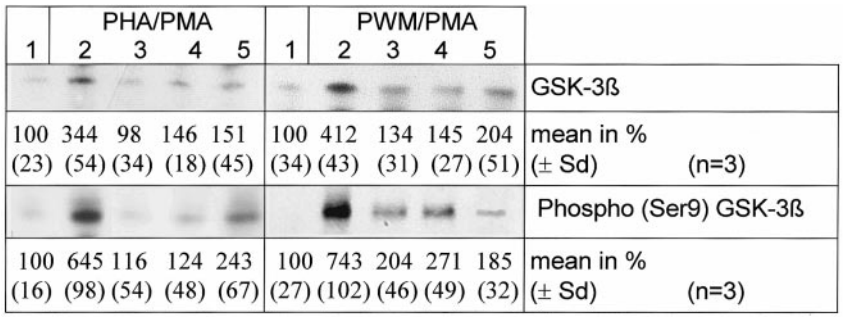

FIG. 2. Quantitative determination of GSK-3β and Phospho-(Ser9)-GSK-3β protein amounts in human T cells. T cells were treated as described in legend to Fig. 1. Compared to resting T cells (lane 1), a strong increase of both total and phosphorylated GSK-3β was observed 24 h after activation (lane 2). This activation-dependent increase is significantly suppressed by the presence of actinonin (lane 3), leuhistin (lane 4), or RB3014 (lane 5) in the culture medium.

Induction of GSK-3β Protein

The induction of GSK-3β mRNA content in activated human T cells is paralleled by a significant increase of GSK-3β protein, as determined by immunoblotting using anti-GSK-3 monoclonal antibodies (Fig. 2). When inhibitors of alanyl-aminopeptidase were applied simultaneously with the T cell activation, GSK-3β protein amounts remained at levels seen in resting T cells. Time-course analyses revealed a steady decline of GSK-3β over a period of three days in response to actinonin (not shown).

Increase of Phospho-(Ser9)-GSK-3β

As observed with total GSK-3β protein, there was also an activation-dependent increase of the amounts of Ser9-phosphorylated GSK-3β. Again, this increase of phosphorylation was significantly suppressed by simultaneous administration of one of the aminopeptidase inhibitors, actinonin, leuhistin, or RB3014 (Fig. 2).

DISCUSSION

The present study is based on previous reports showing that aminopeptidase inhibitors compromise cell proliferation of various cell types [7, 23, 25, 38–42]. The underlying mechanisms and the molecular components employed remained to be identified yet. Recently, MAP kinases Erk1/Erk2 were found to be induced after exposing KARPAS-299 T cells to the aminopeptidase inhibitors actinonin or probestin, giving a first clue on possible down-stream targets of an inhibition of alanyl-aminopeptidase [26]. Subsequently, by applying the ATLAS cDNA expression array (Clontech) and quantitative RT-PCR technique a modulation of Wnt-5a expression by actinonin was shown in peripheral human T cells [27].

The results of this study clearly show an activation-dependent increase in GSK-3β mRNA and protein as well as an increase of phosphorylated GSK-3β. This induction is not restricted to special T cell mitogens, but rather is related to an effective T cell activation. This view is supported by the observation that both PHA and pokeweed mitogen (this study) as well as anti-CD3 mab/interleukin-4 and -9 [27] are capable of inducing GSK-3β mRNA and protein.

Increased Ser9-phosphorylation of GSK-3β, known to inactivate this negative regulatory protein kinase, generally prevents substrates such as β-catenin, eIF2B or cyclin D1 from being phosphorylated and, thus, controls their activity or rescues them from proteasome-mediated degradation [43, 44]. Consequently, there is a stimulation of e.g. TCF-mediated transcription, protein biosynthesis, and cell cycle progression. Dominant negative GSK-3β activates TCF transcription in fibroblasts, but fails to do so in T cells [45]. PHA, as observed also in this study, inhibits GSK-3β activity of human T cells, however, this inhibition is not sufficient to activate TCF-dependent transcription [45]. This may relate to the fact that the activity of GSK-3β towards different substrates is regulated independently by other mechanisms such as the interaction with frequently rearranged in advanced T-cell lymphomas 1 (FRAT1) [46]. In addition to the regulation by Wnt-5a, GSK-3β activity is regulated by PKB, which itself is controlled or modulated by e.g., MAP kinases, PKA, PI3-kinase and, interestingly, signalling from the T cell co-stimulatory molecule CD28. These co-stimulatory signals may well attenuate GSK-3β substrate specifity and play a role for induction of TCF-dependent transcription.

The observed increase of GSK-3β expression and its simultaneous phosphorylation/inactivation requires further investigation. As we analyzed a rather late than an early phase of T cell activation this GSK-3β inhibition might represent already mechanisms limiting activation. As shown by Thomas *et al.* [40], substrate specificity may depend on a "priming" phosphorylation, not necessarily required in later states of activation. Finally, cellular amounts in activated T

cells of active GSK-3β may exceed those of resting cells anyway.

Our data clearly show an activation-dependent induction of GSK-3β expression. When inhibitors of alanyl-aminopeptidase were applied simultaneously with the T cell activation, a significant reduction of GSK-3β expression and activity has been observed. This modulation of GSK-3β expression/activity provides further clues towards a better understanding of the function of leukocyte membrane alanyl aminopeptidase (CD13) and the consequences of its modulation.

It seems difficult to interpret the regulatory role of APN by an intracellular signalling pathway since the enzyme has only a short intracellular tail following the transmembrane domain anchoring it in the plama membrane. However, another possibility could be the regulation through specific processing of a peptide factor acting in an autocrine or paracrine way.

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