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Inhibition of Alanyl Aminopeptidase Induces MAP-Kinase p42/ERK2 in the Human T Cell Line KARPAS-299

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Inhibition of alanyl aminopeptidase (EC 3.4.11.2, aminopeptidase N, CD13) expression, or activity compromise cell proliferation in a number of cell systems [1–6]. The underlying mechanisms and the molecular components involved have not been identified as yet. In this study we show that inhibition of alanyl aminopeptidase enzymatic activity decreases the proliferation rate of the CD13-positive T cell line Karpas-299. By using the ATLAS cDNA expression array (Clontech) we identified the p42/ERK2 MAP kinase as one downstream target of probestin, a potent inhibitor of alanyl aminopeptidase. Probestin and another specific aminopeptidase inhibitor, actinonin, in addition to their capability of inducing erk-2 mRNA levels, significantly increase p42 phosphorylation state. This is the first report on signal transduction components possibly mediating the growth-modulatory effects of alanyl aminopeptidase inhibitors. © 1998 Academic Press

Key Words: **alanyl aminopeptidase, CD13, peptidase, peptidase inhibitor, cell proliferation, ERK2, MAP-kinase, quantitative PCR, Lightcycler.**

Membrane alanyl aminopeptidase (aminopeptidase N, APN, CD13, EC 3.4.11.2) is a 150 kDa metalloprotease constitutively expressed on a wide variety of cells and tissues. The APN gene was cloned in 1989 [7] and subsequently mapped to chromosome 15(q11-qter) [8]. As recently disclosed, the APN gene is spread over 20 exons [9]. APN is involved in the degradation of neuropeptides and cytokines [10–12] and may contribute in extracllular matrix degradation [13]. Moreover, APN functions as receptor for corona virus [14, 15] and CMV [16] and has been suggested to contribute in antigen processing [17]. Within the hematopoietic system, APN is predominantly expressed on cells of the myelo-monocytic lineage [18]. Normal B and resting T cells lack APN expression detectable by standard flow cytometry. However, significant fractions of both acute (B-ALL) or chronic (B-CLL) B cell leukemia show abnormal APN surface expression, which in the case of B-ALL is strongly associated with poor prognosis [19–21]. Elevated APN-mRNA levels and atypical APN-surface expression are frequently observed in human T cell lines and cutanous T cell lymphoma [3, 22, 23]. Mutations in the APN gene may well contribute in the malignant transformation of leukocytes [24]. T cells activated by mitogen *in vitro* or derived from local sites of inflammation *in vivo* show a markedly increased APN gene and surface expression [3, 25–31].

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 [3], of the T cell line H9, and the promyeloic cell line U937 [32]. The aim of this study was to identify molecular players and mechanisms mediating the growth inhibitory effects the aminopeptidase inhibitors, probestin and actinonin, excert on the human T cell line Karpas. By using the ATLAS cDNA expression array (Clontech) we identified ERK2/p42 MAP kinase as a downstream target of APN inhibition. These primary data were confirmed by quantitative PCR and immunoblotting.

MATERIALS AND METHODS

Reagents. The ATLAS cDNA expression array was from Clontech (Heidelberg, Germany). ^{32}P-dATP (6.000 Ci/mmol) was purchased from NEN Life Science Products (Bruexelles, Belgium).

Cell culture. The human T cell lymphoma cell line KARPAS-299 (DSM ACC 31) was purchased from DSM (Braunschweig, Germany). Cells were grown in RPMI 1640 culture medium supplememted with 10% fetal calf serum (Gibco BRL, Eggenstein, Germany) in 50 ml culture flasks (Falcon, Germany) to densities of approx. 10^6 cells/ml. Cells were cultured in the presence of 50 μmol/ml probestin (kind gift from T. Aoyagi [33]) or actinonin (Sigma, Deisenhofen, Germany) for different lenght of times (see legend to figures.)

RNA-isolation. For using the ATLAS cDNA expression array, total RNA was prepared prior to the enrichment of polyA$^+$-RNA. Total RNA was prepared essentially according to Chomczynski and

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Sacchi [34]. Briefly, cultured KARPAS cells (5 x 10^7 cells) were washed twice in PBS and immediately homogenized in 3 ml of ice-cold Trizol LS (Gibco BRL, Eggenstein, Germany). After the addition of 600 μl chloroform and a 15minute centrifugation (10.000 x g, 4°C) RNA was ethanol-precipitated from the aqueous phase. Total RNA was resuspended in 100μl of RNase-free water and polyA$^+$-RNA was prepared using the Oligotex-mRNA midi kit (Qiagen, Hilden, Germany) exactly following the recommended protocol. The amount of polyA$^+$-RNA was determined spectrophotometrically using the Genequant (Pharmacia, Munich, Germany). One μg of polyA$^+$-RNA was used for cDNA-synthesis.

Total RNA for use in the RT-PCR was prepared from 2 x 10^7 cells using the RNeasy kit (Qiagen, Hilden, Germany). RNA was treated with 1U/μg RNA of DNase I (RNase-free, Boehringer Mannheim, Mannheim, Germany) for 30 min at 37°C prior to a second round of purification by means of the RNeasy kit. RNA concentration was determined as described for polyA$^+$-RNA.

ATLAS cDNA expression array. For analyzing different gene expression in untreated and probestin-treated KARPAS-299 cells the ATLAS cDNA expression array (Clontech) was used. The materials provided with the kit were used and the recommended protocol was followed in all steps, except that α-^{32}P-dATP with a higher specific activity (6.000 Ci/mmol) was used. Briefly, in each case 1 μg polyA$^+$-RNA were converted into ^{32}P-labelled first strand cDNA by means of MMLV reverse transcriptase. Unincorporated ^{32}P-labelled nucleotides were removed by CHROMA SPIN-200 column chromatography. cDNA-fractions of highest activity were pooled and hybridized to one of the ATLAS membranes. After prehybridization (30 mins at 68°C in ExpressHyb (Clontech) supplemented with 200 μg/ml herring sperm DNA (Sigma, Deisenhofen, Germany), the heat denatured probe was added. Hybridization occurred overnight at 68°C. Membranes were washed 4 x 20 mins in 2 x SSC/1% SDS at 68°C followed by two washes in 0.1 x SSC/0.5% SDS (20 min, 68°C). Membranes were sealed in sample bags (LKB Pharmacia) and exposed to X-ray film for 1 to 3 days.

Quantitative PCR. Two microgram of total RNA were converted into first strand cDNA using ReadyToGoYouPrime beads (Pharmacia, Munich, Germany) and random hexanucleotides (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions. One microliter of the resulting 100μl cDNA mixture was used in a typical PCR experiment.

Quantitative PCR was performed in 10μl reaction cuvettes using the Lightcycler LC24 (Idaho Technology, Boston, USA). For the determination of erk2 mRNA content, a 10 μl reaction mixture consisted of 1 x reaction mixture with BSA (Idaho Technology), 3 mM $MgCl_2$, 200 μmol dNTP, 0.4 U InViTaq polymerase (InViTec, Berlin, Germany), 0.2 μl of a 1:1000 dilution of SYBR Green I (Molecular Probes, USA), and 0.5 μmol of the erk-2 specific primers erk2-US 5′-CATCGCCGAAGCACCATTCAAG and erk2-DS 5′-GATAAGCCAAGACGGGCTGGAG. Initial denaturation at 95°C for 10s 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 16 s. The fluorescence intensity of the double-strand specific SYBR Green I, reflecting the amount of formed PCR-product, was read at the end of each elongation step. GAPDH-mRNA amounts were determined using the RT primer pair commercially available from Stratagene (Heidelberg, Germany) and used to normalize the sample cDNA content.

Cell proliferation assay. KARPAS-299 cells were seeded into 96well plates at a density of 10.000 cells/100μl culture medium/well. Probestin or actinonin, respectively, were added at 50 μmol/l. After 3 days of culture cell number was determined by using the WST-1 cell proliferation kit (Promega, Heidelberg, Germany) following the recommended protocol.

Phospho-p42 Western blotting. After reaction times given in the legends to the figures aliquots containing 2x10^6 cells were collected and the cells were subsequently spun down. The cell pellets were allowed to lyse within 10 mins in 50 μl lysis buffer (20 mM TRIS-HCl, pH 6,8, 0,2% (v/v) Triton X-100, 100 mM KCl, 1 mM MgCl2, 10 mM EGTA, 10 mM NaF, 1 mM Na-vanadate, 1 μM Calyculin A) on ice. Extensive controls revealed that under the conditions applied nuclei were completely disintegrated (data not shown). Lysates were centrifuged (100,000 x g, 2°C, 30 min) and 40 μl of each supernatant was mixed with 10 μl 5x sample buffer and subsequently boiled for 5 min. 10 μl aliquots of each sample were separated in a 10% SDS-polyacrylamide gel. After transferring the proteins onto nitrocellulose activated p42/erk1 was detected by means of rabbit-anti-P-p42 purified Ig (specificity: Phospho-Thr202/Tyr204, New England BioLabs, USA) and goat-anti-rabbit-POD/UltraSignal chemiluminescent substrate (Pierce, The Netherlands).

RESULTS

Probestin and actinonin suppress KARPAS-299 cell proliferation rate. In the presence of 50 μmol/l of the aminopeptidase inhibitors probestin or actinonin, respectively, there was a significant reduction of KARPAS-299 cell number after 3 days of culture, compared to cells grown in medium only. Probestin decreased the cell number to 50,3% and comparable effects were seen with actinonin (52,1% of control) (Fig. 1). Since there was no significant loss of cell viability 3 days after the administration the inhibitors observed, a decrease in cell number due to cytotoxic properties of the inhibitors could be excluded.

ATLAS cDNA expression array. Using the ATLAS cDNA expression array (Clontech) it was found, that exposure of KARPAS-299 cells to 50 μmol/l probestin over a 20 hour period resulted in significant changes of gene expression in comparison to untreated cells. One of the most prominent changes observed was an increase of erk2 mRNA (Fig. 2, position B5n of the ATLAS) in probestin-treated cells.

Quantitative PCR. Using the Lightcycler LC24 (Idaho Technology) the aminopeptidase inhibitor-dependent induction of erk2 mRNA content in KARPAS-299 cells could be confirmed by quantitative PCR. Primers and PCR conditions were as outlined in the "Materials and Methods" section. Furthermore, the time course of the inhibitor-dependent erk-2 mRNA

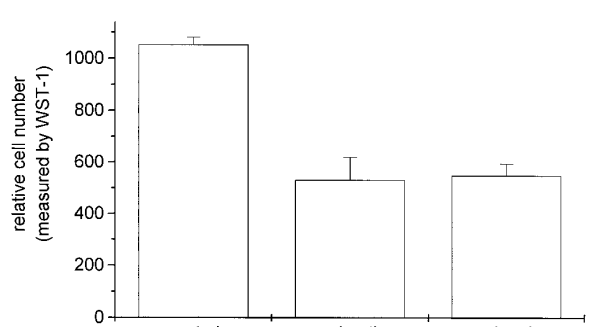

FIG. 1 Measurement of KARPAS-299 cell number after 3 days of culture in medium only (control) or in the presence of 50 μmol/l of probestin or actinonin, respectively. Cell number was determined by using the WST-1 cell proliferation kit (Promega).

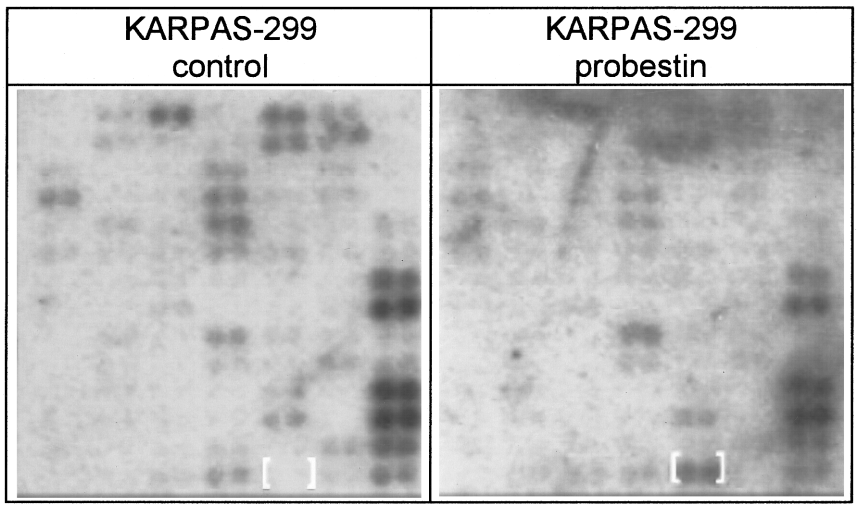

FIG. 2. Differential gene expression after exposure of KARPAS-299 cells to probestin. Expressed mRNAs of untreated or probestin-treated (20 hours, 50 μmol/l probestin) cells were compared by hybridization of ^{32}P-dATP labelled cDNA to the ATLAS human cDNA expression array I (Clontech). Shown is quadrant B of the ATLAS screen with the probestin-dependently induced erk2-mRNA (position B7n) in brackets.

induction was followed: already 4 hours after the administration of probestin or actinonin, respectively, there was an increase of erk2 mRNA detectable (183 % or 216% of control). Maximum expression was observed after 24 hours (332% or 324% of control), whereafter the erk2 mRNA content steadily decreased (Fig. 3).

Induction of p42/ERK2 protein expression and phosphorylation. The induction of erk2 mRNA content in KARPAS-299 cells by probestin and actinonin is paralleled by a weak increase of p42/ERK2 protein after 8 to 24 hours, as determined by immunoblotting using anti-p42 polyclonal rabbit antibodies (not shown). Much more striking was the increase in the amount of activated, phosphorylated ERK2, Pp42, as detected by anti-Pp42 polyclonal antibodies. Results of a representative experiment were shown in Figure 4. Our immunoblot analyses revealed a maximum increase of Pp42 8 hours after the administration of actinonin or probestin. Densitometry scanning of the chemoluminographs (top of Figure 4) revealed an increase by 7,23-fold after 8 hours of actinonin treatment, whereas probestin provoked a more than 3-fold increase in Pp42 levels. 24 hours after inhibitor administration the amounts of the activated (phosphorylated) ERK2 returned to base level.

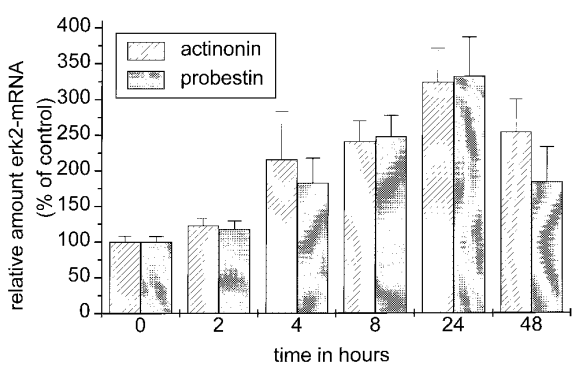

FIG. 3. Quantitative determination of erk2-mRNA contents in KARPAS-299 cells. Cells were treated with probestin or actinonin for the times indicated and their relative erk2-mRNA content was analyzed by quantitative PCR using the Lightcycler LC24 (For details see the Materials and methods section).

DISCUSSION

The present study based on previous reports showing that aminopeptidase inhibitors compromise cell proliferation of various cell types [1–6]. The underlying mechanisms and the molecular components employed remained to be identified yet. Therefore, the CD13-positive KARPAS-299 cell line was used as a model to identify potential cellular targets mediating the growth inhibitiory effects of the aminopeptidase inhibitors, probestin and actinonin.

Since it has not been shown before, whether these inhibitors effect proliferation of KARPAS-299 cells, we first addressed this question by using the WST-1 proliferation kit. As observed with other cell types mentioned above, probestin and actinonin were found to suppress KARPAS-299 cell proliferation to a significant extent. At the concentration used in this study both inhibitors are supposed to preferentially affect alanyl aminopeptidase [33, 35, 36].

The ATLAS cDNA expression array (Clontech) was used for identifying potential targets of the probestin-mediated inhibition of alanyl-aminopeptidase. A 20hour exposure of KARPAS-299 cells to probestin provoked selective changes in the mRNA expression pat-

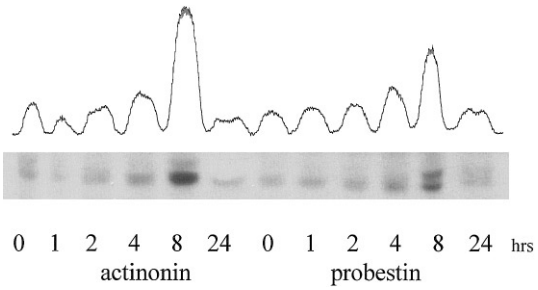

FIG. 4. Activation of p42/MAP kinase in KARPAS-299 cells by probestin or actinonin as determined by immunoblotting of Pp42. Bottom: Chemoluminograph of a representive experiment shows the peak activation of ERK2 after 8 hours. Top: Densitometry scanning revealed a 7-fold increase by actinonin and a 3-fold increase by probestin of Pp42 amounts. Note, that there is also a faint activation of p44 MAP kinase after 8 hours (upper band).

tern. One of the most prominent changes observed was the induction of erk2 mRNA, which has been confirmed by quantitative PCR. Using this technique we also showed that maximum erk2 mRNA levels occur about 24 hours after the administration of probestin. In addition, we observed an increase of p42/ERK2 protein and, especially, of the activated phosphorylated Pp42 MAP kinase. Most notably, maximum levels of activated Pp42 MAP kinase were detected after 8 hours. This discrepancy between induction of erk2 mRNA and activation of p42 MAP kinase strongly suggests that both mechanisms apply in the cell system studied, but function independently of each other. To our knowledge, this is the first report on both transcriptional and post-translational regulation of lymphocyte p42/ERK2 expression and activation in response to CD13-ligandation. As it is obvious from figure 4, 8 hours after the administration of actinonin and, especially, probestin, there is a slight, but significant activation of p44 MAP kinase, too. This finding, however, has to be substantiated by further analyses.

Protein kinase signalling pathways are important mediators of signaling responses in all eukaryotes. The extracellular signal-regulated kinase (ERK) cascade was the first mitogen-activated protein kinase (MAPK) system characterized in mammalian cells [37]. ERK2, together with ERK1, is the best characterized member of the MAPK subfamily and its cDNA was one of the first to be cloned [38–40]. The signaling cascades leading to ERK2 activation are exceptionally well described [41]. Activation of ERK2/p42 occures rapidly via phosphorylation, peaking 5 mins after receptor-ligandation and is often transient in nature. Sustained activation (up to 1 hour) in a number of systems results in nuclear translocation and subsequent changes in gene expression and cellular function [42]. Inhibition of alanyl aminopeptidase by probestin or actinonin does not lead to an immediate activation/induction of p42/Erk2. Instead, peak ERK activation occurred more than 8 hours after inhibitor administration and was clearly sustained in nature. At present there is no answer at hand to explain this delayed activation of Erk2 and its gene transcription. However, it is tempting to speculate that due to the inhibition of membrane alanyl aminopeptidase activity there is a slowly ongoing accumulation of a (peptide) receptor ligand, eventually triggering the MAP kinase pathway upon association with an extracellular receptor. Alternatively, inhibition of membrane alanyl aminopeptidase activity might cause some modulation of intracellular signalling components resulting in changes of ERK levels, resembling the situation in rat brain after long-term administration of morphin [43–46].

Considering the growth inhibitory effects of aminopeptidase inhibitors, our finding of an induction of ERK2 expression/activity was quite unexpected. However, the lack of significant cell death after inhibitor administration could relate to the fact, that the employment of the MAP kinase pathway prevents KARPAS-299 cells from undergoing apoptosis. Interestingly, coupling of inhibitory receptor ligands to ERK activation has been described recently by Gutstein et al. [43] and Chuang and co-workers [47] for the opioid receptor system. On the other hand, our data do not exclude the possibility that other downstream targets than ERK2 cause the growth inhibition.

In conclusion, we found an induction of Pp42 ERK2 and an increase of Erk2 mRNA amounts in KARPAS-299 cells 8 hours after the administration of probestin or actinonin, potent inhibitors of alanyl aminopeptidase. This is the first report on signal transduction events in response to the modulation of alanyl aminopeptidase activity. An important issue that has to be addressed in future studies is how alanyl aminopeptidase-derived signal is transduced to the MAP kinase pathway. The data presented so far provide clues to help us better understand the function of leukocyte membrane alanyl aminopeptidase (CD13) and the consequences of its modulation.

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