Pergamon

Bioorganic & Medicinal Chemistry, Vol. 4, No. 7, pp. 1113–1119, 1996

PII: S0968-0896(96)00104-6
0968-0896/96 $15.00+0.00

Catabolism of the Hemoregulatory Peptide *N*-Acetyl-Ser-Asp-Lys-Pro: a New Insight into the Physiological Role of the Angiotensin-I-Converting Enzyme *N*-Active Site

Anne Rousseau-Plasse,* Maryse Lenfant and Pierre Potier
Centre National de la Recherche Scientifique, Institut de Chimie de Substances Naturelles, 91198 Gif sur Yvette, Cedex, France

Abstract—The tetrapeptide *N*-Acetyl-Ser-Asp-Lys-Pro (AcSDKP) was first isolated from bone marrow extracts and shown to be involved in the negative control of hematopoiesis by preventing the recruitment of primitive stem cells into S-phase. In vitro studies on AcSDKP catabolism in human plasma revealed that AcSDKP was cleaved by plasmatic angiotensin-I converting enzyme (ACE). The evaluation of the respective involvement of the two active sites of ACE in AcSDKP degradation in vitro revealed that the *N*-active site was preferentially involved in this catabolism. Moreover, an in vivo study on healthy volunteers of the catalytic efficiency of ACE towards AcSDKP after administration of Captopril® demonstrated that AcSDKP was a physiological substrate of ACE. AcSDKP might represent the first natural specific substrate of the *N*-active site of the enzyme. These results pose the question of a potential role of ACE in the control of hematopoiesis as well as possible applications of ACE inhibitors to cope with dysfunctions in which AcSDKP might exert physiological control. Copyright © 1996 Elsevier Science Ltd

Introduction

Hematopoiesis might be defined as a dynamic process involved in the continuous and regulated production of blood cells. Mature blood cells are derived from immature precursors, through complex steps of multiplication and differentiation. The ultimate marrow and blood repopulating ability depends on a pluripotent hematopoietic stem cell (HSC), which has the capability of self-renewing and differentiating into the whole myelolymphopoietic lineages. Therefore, the HSC is able to reconstitute the long-term hematopoiesis in a lethally irradiated host. The hematopoietic system is thus organized in a hierarchical way, at the origin of which the HSC multiply and differentiate to give rise to the multipotent progenitors, which in turn lead to lineage-restricted progenitors that have lost their self-renewal capability and are further differentiated into the first morphological recognizable cells characteristic for each blood lineage, leading to mature blood cells formation (Fig. 1).[1] The regulation of hematopoiesis at the levels of stem, progenitor, precursor, and mature cells is mediated by a balanced response to competitive stimulatory and suppressing influences induced by the medullar microenvironment and multiple stimulatory and inhibitory factors.

The microenvironment, which consists of different cell populations (the stromal cells) such as macrophages, fibroblasts, endothelial cells, adipocytes, and molecules of the extracellular matrix (MEC), is implicated in complex interactions with the HSC.[2] Those interactions involve multiple cell-surface receptors present on stromal cells, the extracellular matrix molecules and soluble or membrane-bound stromal-cell-derived regulatory factors (Li et al., personal communication[3]). Various factors provide positive and negative signals, leading to a balanced regulation of the proliferation and differentiation processes of the hematopoietic cells. Research on stimulatory factors and depiction of their genes have made spectacular progress during the past decade due to the development of molecular biology and experimental procedures allowing in particular the in vitro culture of the progenitor cells.[4] Difficulties in demonstrating the biological activity of inhibitory factors has slowed down the research in this field. However, several molecules presenting inhibitory activity on the hematopoietic system have now been identified: besides high-molecular weight proteins that display pleiotropic effects on various hematopoietic lineages, such as interferons (INFs), tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), and macrophage inflammatory protein-1α (MIP-1α), various metabolic regulators such as acidic isoferritin, lactoferrin, prolactin, and low-molecular weight factors have been shown to exert regulatory functions on precursor cell proliferation.[6,7] Recently, two peptides have been identified: the pentapeptide <Glu-Glu-Asp-Cys-Lys (pEEDCK), an inhibitor of the granulomacrophagic lineage,[8] and the tetrapeptide *N*-Acetyl-Ser-Asp-Lys-Pro (AcSDKP), an inhibitor of the entry into S-phase of the myeloid and erythroid quiescent progenitors.[9,10] Recently, our laboratory has investigated the catabolism of this molecule.

Key words: hematopoiesis, AcSDKP, angiotensin-I-converting enzyme (ACE), angiotensin-I-converting enzyme inhibitor.
Abbreviations: <Glu, pyroglutamic acid.

The hemoregulatory peptide *N*-Acetyl-Ser-Asp-Lys-Pro

In 1989 the structure of the tetrapeptide AcSDKP was determined and its synthesis realized.[9] This molecule, isolated from fetal calf bone marrow, has been identified as the factor detected in the bone marrow extracts and is able to inhibit in vivo the entry into DNA synthesis of the hematopoietic stem cells triggered into S-phase by γ-irradiation treatment.[11] In humans, AcSDKP has been localized in different tissues, cells, and biological fluids; the tetrapeptide was detected in the largest concentration in the spleen compared to the intestine, the thymus and the stomach.[12] In the bone marrow, AcSDKP was recently shown to be stored by the MEC and synthesized by the macrophages, whereas the stromal cells might be implicated in its catabolism (Li et al., personal communication). AcSDKP was also detected in the serum and plasma, as well as in the blood mononucleated cells and particularly in lymphocytes and macrophages.[13] This ubiquitous nature of AcSDKP asked the question of its biological role.

AcSDKP has been shown to act on quiescent murine hematopoietic progenitors by preventing in vivo their recruitment into S-phase.[9,14] In vitro, the peptide inhibited the cycling of murine and human myeloid and erythroid progenitors induced by stimulators in long term bone marrow culture as well as in semi-solid assays.[15–24]

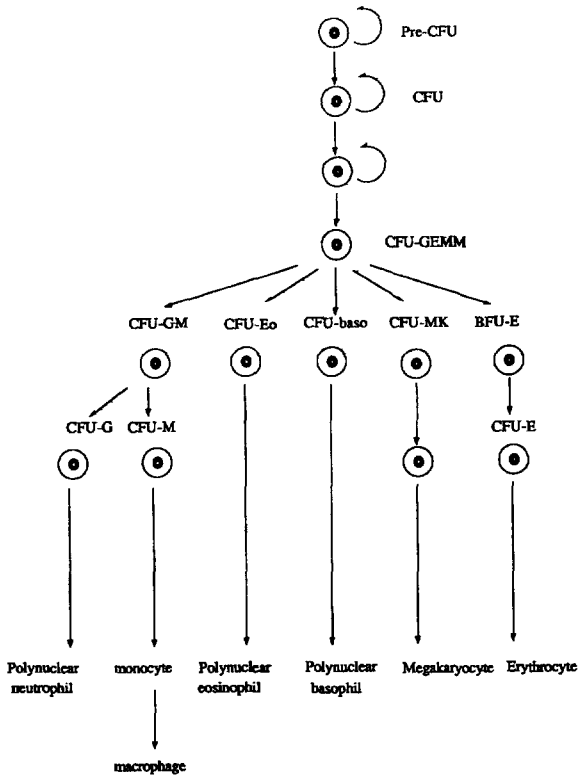

Figure 1. The hematopoietic system is organized in a hierarchical way and is schematically represented by: (1) the hematopoietic stem cells (HSC), which multiply and differentiate to give rise to (2) the multipotent progenitors, which in turn lead to lineage-restricted progenitors, that are further differentiated into (3) the first morphologically recognizable cells characteristic for each blood lineage, leading to the formation of mature blood cells. CFU, colony forming unit; BFU, burst forming unit; G, granulocyte; M, macrophage; Eo, eosinophil; Baso, basophil; MK, megakaryocyte; E, erythrocyte.

Furthermore, as previously described for the dialysable fraction, AcSDKP administered in mice protected in vivo the hematopoietic stem cells from the cytotoxicity of phase-dependent drugs and irradiation, leading to an increased survival of mice treated with lethal doses of cytosine arabinoside or cyclosphosphamide, and irradiation.[25–27] This protective effect of AcSDKP has been confirmed in vitro on human cells treated with 4-hydroxyperoxycyclophosphamide or 3′-azido-3′-deoxythymidine.[28,29] Positive results obtained in preliminary clinical assays (Phases I–II) in cancer patients suggest applications of AcSDKP in human clinical treatment (Beaufour, personal communication[30]).

Finally, AcSDKP acted in vitro on medullar stromal cells by enhancing the adherence of hematopoietic stem cells to stromal cell lines, as well as decreasing stromal cell proliferation.[31–33] This implication of AcSDKP in the adherence processes has been confirmed in a rosettes formation assay formed between sheep erythrocytes and Jurkatt cell line.[34] Those experiments revealed the crucial role of the tripeptidic sequence SDK, which appeared essential for the activity of the molecule.[20] Furthermore, the antiproliferative activity of AcSDKP demonstrated in long-term bone marrow culture, on the progenitors present in the adherent layer could not be evidenced in the absence of stromal cells, suggesting that AcSDKP activity was mediated by accessory cells of the microenvironment.[19,35] The necessity of auxiliary cells for AcSDKP activity might explain the fluctuations or the absence of AcSDKP activity in in vitro models, implicating either purified bone marrow cells ($CD34^+$), or hematopoietic and transformed cells in a semi-solid environment.

The biological functions of AcSDKP on normal hematopoiesis, as well as the absence of antiproliferative activity of AcSDKP on leukemic cells have suggested its clinical use in anticancer treatments.[25,30] Studies on the catabolism of the molecule conducted in our laboratory have demonstrated that, in human plasma, the soluble angiotensin-I-converting enzyme was responsible for the limiting catabolic step of AcSDKP hydrolysis.[36]

Angiotensin-I-converting enzyme

Angiotensin-I-converting enzyme (ACE; peptidyl dipeptidase A, kininase II, EC 3.4.15.1) is a zinc-dipeptidyl carboxypeptidase, which also displayed endopeptidase activity. The primary specificity of ACE was to cleave *C*-terminal dipeptides from oligopeptide substrates presenting a free *C*-terminus and devoid of either a penultimate proline residue or a terminal dicarboxylic amino acid; this explains the major physiological function of ACE in the conversion of angio-

tensin-I into the potent vasoconstrictor angiotensin-II.[37] As shown by in vitro assays, this function might be enhanced through the inactivation of the vasodilator bradykinin, which was cleaved by the sequential removal of two *C*-terminal dipeptides.[38] These activities explained the predominant role of ACE in the regulation of blood pressure. Besides this dipeptidyl-carboxypeptidase activity, ACE was endowed with an endopeptidase activity, which has been observed in vitro on substrates that are *C*-terminal amidated such as substance P, where the enzyme cleaved the *C*-terminal tripeptide amide.[39,40] The in vitro endopeptidase activity was also functional on the *N*-terminal part of Luteinizing hormone-releasing hormone (LH-RH), where ACE cleaved, besides the *C*-terminal amidated tripeptide, the *N*-terminal <Glu tripeptide.[41]

The cDNA sequencing of ACE has been carried out in humans and showed that the somatic isoform of ACE was derived from a duplicated ancestral gene that led to a 150–180 kD enzyme, which contained the two homologous parts of the original protein.[42,43] Each domain comprised the consensus sequence His-Glu-X-Y-His associated with one zinc atom, characteristic of the metallo-binding active sites of the peptidases of the Gluzincin family.[43,44] Somatic isoforms occurred as insoluble enzymes cell-membrane bound through their *C*-terminal domain.[42,45,46] Somatic ACE isoforms presented 17 different sites of glycosylation, which were differently substituted depending on the tissue origin.[42,47] Following a post-transcriptional enzymatic cleavage, circulating soluble forms were found in plasma.[45,46,48,49] Besides those high-molecular weight somatic enzymes, a 90–110 kD insoluble ACE was found in spermatids and spermatozoids, associated with the cell membranes. In this case, a unique active site was present, located in the *C*-terminal domain.[50–52] Concurrently, a truncated soluble 100 kD *N*-terminal isoform of ACE has been detected in the ileal fluid.[53] In the case of the testicular *C*-terminal ACE, it has been established in mammals that the enzyme was derived from a gene identical to that coding for the sequence of the somatic enzyme, which is transcripted from a different initiation site located in an intron corresponding to a noncoding sequence of the cDNA of the somatic isoform of ACE.[43,50,51,54–57] No information was yet available concerning the gene coding for the ileal *N*-terminal ACE. Recently, a soluble *C*-terminal isoform of ACE has been discovered in *Musca domestica*, different from the mammalian counterparts. It appeared to be derived from a conserved and nonduplicated gene, restricted to the *C*-terminal domain of the enzyme, which has escaped to the duplication process observed in mammals.[58–60]

Conversely to the results related in the previous reports, it was recently established that both sites of the somatic ACE were functional.[46] Further studies demonstrated that both domains exhibited similar catalytic activities towards angiotensin-I, bradykinin, and substance P.[61,62] However, the two active sites differed by the level of chloride ion concentration necessary to potentiate their maximal activities; whereas the *N*-active site was maximally activated at chloride concentrations varying from 50 to 100 mM and was inhibited at supra-optimal chloride concentrations, the *C*-active site reached its optimal activity at chloride concentrations above 300 mM.[61,62] Furthermore, ACE inhibitors displayed different potencies towards the two active sites of ACE.[63] Finally, whereas both *N*- and *C*-terminal catalytic domains were equally involved in the conversion of angiotensin-I and the degradation of bradykinin through a dipeptidyl carboxypeptidase activity, the in vitro *N*-terminal endopeptidasic cleavage of LH-RH seemed to be restricted to the *N*-terminal active site.[61] However, until now, no evidence for an implication of ACE in the in vivo cleavage of LH-RH has been presented; furthermore the values of the catalytic constants ($k_{cat}/K_m = 0.011\ \mu M^{-1}\ s^{-1}$) and substrate affinity ($K_m = 760\ \mu M$) determined in vitro for LH-RH and the full-length *N*-active site of ACE were such that the possibility that LH-RH might be considered as a physiological substrate of the *N*-active site of ACE can be questioned.[61]

Implication of the *N*-terminal active site of ACE in AcSDKP degradation

In preliminary studies carried out in our laboratory, several lines of evidence established the involvement of human plasma ACE in the limiting step of the in vitro degradation of AcSDKP.[36] In order to determine precisely which of the two active sites of ACE was involved in AcSDKP degradation in vitro, a study was undertaken using an analogue of AcSDKP, specifically radiolabeled on the lysyl side chain ^3H AcSDKP,[64] and the wild-type recombinant and the two full-length mutants of ACE, which were developed in the laboratory of P. Corvol (Collège de France).[46] Each mutant contained one of the two sites inactivated by substitution of the two zinc-binding histidyl residues with lysyl residues in the consensus binding sequence His-Glu-X-Y-His; thus, either the *C*- or the *N*-domain was presented as functional. Kinetic studies indicated that whereas the *N*- and *C*-active sites of ACE were able to cleave AcSDKP by a dipeptidase activity, the two sites were differently involved in this degradation as they displayed markedly different kinetic parameters and different responses to chloride ions and ACE inhibitors.

Whereas the hydrolysis of AcSDKP by the wild-type recombinant ACE and the *N*-active mutant were slowly activated by chloride ions, the *C*-active mutant underwent an increased sensitivity to this anion (Table 1). Marked differences were observed in the values of the catalytic constants of the *N*-active ($K_m = 30\ \mu M$, $k_{cat}/K_m = 0.4\ \mu M^{-1}\ s^{-1}$) and the *C*-active ($K_m = 21\ \mu M$, $k_{cat}/K_m = 0.01\ \mu M^{-1}\ s^{-1}$) mutants, indicating that, despite AcSDKP displaying a similar and strong affinity for the two active mutants of ACE, the peptide was cleaved 40 times faster by the *N*-active than the *C*-active site of ACE; furthermore, the catalytic efficiency of the wild-

Table 1. Chloride dependence of AcSDKP hydrolysis by recombinant forms of ACE

ACE	K'_a[a] (mM)
Wild-type ACE	5
N-active ACE	5
C-active ACE	29

[a]K'_a: activation constant. Values are taken from ref 66.

type ACE (K_m=39 μM, k_{cat}/K_m=0.3 μM^{-1} s^{-1}) was similar to that of the *N*-active mutant.[65] In agreement with these results, a monoclonal antibody, specifically directed against the *N*-active site of ACE, exhibited an inhibitory potency on the wild-type recombinant ACE towards AcSDKP hydrolysis similar to that observed on the *N*-active mutant of ACE and significantly stronger than that observed on the *C*-active mutant. Finally, different ACE inhibitors were studied for their potency to inhibit the degradation of AcSDKP and Hip-His-Leu (Cushman substrate), peptides respectively specific for the *N*-active site and the *C*-active site of ACE. Results indicated that the relative inhibitory potency of those ACE inhibitors depended on the specificity of the substrate for the active site studied: whereas trandolaprilat showed the highest inhibitory potency for both active sites independently of the substrate studied, lisinopril showed a greater inhibitory potency than captopril on the activity of the *N*-active site when AcSDKP was used as a substrate, reverse results being observed when Hip-His-Leu was used as substrate. The *C*-active mutant led to comparable data.

It is worth noting that *angiotensin* AcSDKP hydrolysis by ACE was characterized by kinetic constants in the range of those obtained for angiotensin-I, a physiological ACE substrate,[62] conversely to the kinetic data obtained for LH-RH hydrolysis (Table 2).[61] These observations suggested that AcSDKP might represent the first potential physiological substrate specific for the *N*-active site of ACE.

Our second goal was to evaluate the in vivo implication of ACE in AcSDKP catabolism. A study was undertaken, in which captopril, an ACE inhibitor, was administered to healthy volunteers. The acute in vivo ACE inhibition induced by a single oral dose of captopril (50 mg) was quantified by determining the plasma levels of angiotensin-I, angiotensin-II, and renin activity. The endogenous AcSDKP levels in plasma were measured by using the enzyme-immunoassay previously described,[13] and the plasmatic ACE activity was determined by measuring the in vitro catabolism of [^{3}H]AcSDKP and Hip-His-Leu, the latter being the standard substrate routinely used in the quantification of ACE activity. After captopril intake, results showed an increase in AcSDKP level in vivo, which was correlated with a decrease in the in vivo physiological ACE activity. When plasmatic ACE activity towards AcSDKP was measured in vitro, similar inhibition was observed (Fig. 2). In agreement with previous observations, results suggested that ACE was the major enzyme implicated in the tetrapeptide degradation in vivo. These results established that AcSDKP might represent the first physiological substrate specific for the *N*-active site of ACE. Moreover, the profile of ACE inhibition as assessed by the hydrolysis of AcSDKP in vitro was very close to that observed for both endothelial and plasmatic ACE inhibition evaluated by the time course evolution of the in vivo angiotensin-II/angiotensin-I ratio, but differed from Hip-His-Leu hydrolysis measured in vitro in plasma of captopril-treated volunteers (Fig. 3).[66] Therefore, measurement of AcSDKP hydrolysis in vitro in the plasma of treated volunteers might provide an accurate marker of the in vivo activity of physiological ACE.

Perspectives

The role of angiotensin-I-converting enzyme (ACE) in the renin–angiotensin system (RAS), implicated in the maintenance of blood pressure and fluid and electrolyte homeostasis, is now well established and supported by the important colocalization of ACE with the elements of the RAS in endothelial and epithelial cells of numerous tissues such as blood vessels, kidney, adrenals and heart.[67] However, the discovery of this enzyme in different cells and tissues such as renal proximal tubular cells, monocytes, brain and reproductive organs, along with its broad substrate specificity, has led to the hypothesis that, besides its important role in the maintenance of cardiovascular homeostasis, ACE might be implicated in a number of other physiological processes such as the catabolism of neuropeptides, immunity and reproduction.[68]

Table 2. Comparison of the kinetic constants obtained with recombinant forms of ACE for the hydrolysis of AcSDKP, angiotensin-I and LH-RH

ACE	AcSDKP[a]		Angiotensin-I[b]		LH-RH[c] terminal tripeptide	
	K_m (μM)	k_{cat}/K_m (μM^{-1} s^{-1})	K_m (μM)	k_{cat}/K_m (μM^{-1} s^{-1})	K_m (μM)	k_{cat}/K_m (μM^{-1} s^{-1})
Wild-type ACE	39	0.3	16	2.5	265	0.007
N-active ACE	30	0.4	15	0.7	760	0.002
C-active ACE	21	0.01	18	1.9	520	0.002

[a]Values taken from ref 66.
[b]Values taken from ref 63.
[c]Values taken from ref 62.

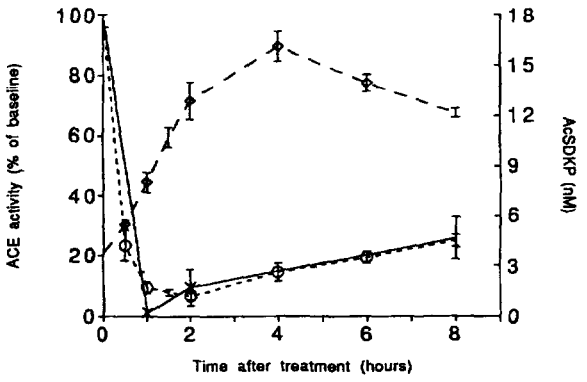

Figure 2. Comparison of the time-course evolution of ACE inhibition estimated in vivo using the angiotensin-II/angiotensin-I ratio (AII/AI) (×) and in vitro using [^{3}H]AcSDKP as substrate (○), and of endogeneous plasma AcSDKP levels (◇) (mean ± SEM). Data are taken from ref 67.

Much insight into the primary structure of ACE has been provided by recombinant techonology, which pointed out the presence of the two active sites in the somatic form of this enzyme.[42] The testicular and ileal ACEs, however, exhibited a single catalytic site, each respectively located in the *C*- and *N*-terminal part of the proteins.[50,51,53] Whereas, the functions of the *C*-active site have been extensively studied and its role recognized in the metabolism of numerous peptides such as the formation of angiotensin-II or the catabolism of substance P and bradykinin,[61,62] the physiological role of the *N*-active site of ACE, up to now, was still questioned. The evidence presented here, which established that the *N*-catalytic site was specifically involved in the catabolism of the hemoregulatory peptide AcSDKP, opened a new field of investigation concerning a potential role of ACE in the regulation of the hematopoietic system. The tetrapeptide was shown to inhibit the entry into S-phase of the pluripotent hematopoietic stem cell, and a fine tuning of the peptide concentration via ACE activity at the level of the bone marrow might implicate this enzyme in the hematopoietic hemostasis. Such hypothesis is supported by the occurrence of anemia observed following the administration of ACE inhibitors, in patients undergoing renal failure or after renal transplantation.[69,70]

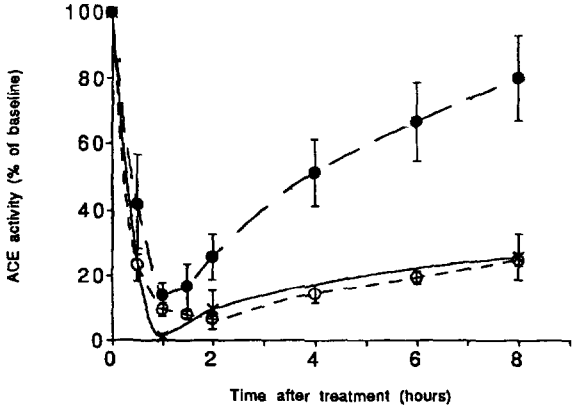

Figure 3. Comparison of the time course evolution of ACE inhibition estimated in vivo using the angiotensin-II/angiotensin-I ratio (AII/AI) (×), and measured in vitro using Hip-His-Leu (●) and [^{3}H]AcSDKP (○) as substrates (mean ± SEM). Data are taken from ref 67.

The physiological role of AcSDKP is still far from being fully elucidated, and the detection of this peptide in numerous organs in which ACE has been localized is worth pointing out.[13,71–73] AcSDKP activites are not restricted to the hematopoietic system, as suggested by its inhibitory effect on hepatocytes entry into S-phase;[74] a possible role in the inhibition of pathological hyperplastic processes has been suggested recently.[66] The possibility that ACE might be implicated in such functions via AcSDKP catabolism is open to question. Recently, high concentration of the peptide has been detected in testicular specific cell populations (Dr J. Bakala, personal communication). This observation might be correlated with the uniqueness of the testicular ACE structure, which is characterized by the absence of the *N*-active catalytic domain responsible for AcSDKP catabolism.[50,51] Such a phenomenon might influence spermatogenesis regulation. No information is yet available concerning the concentration of AcSDKP at the level of the ileal fluid, where a specific *N*-active ACE has been detected;[53] such an investigation might be worthwhile in order to assess a possible physiological role for AcSDKP at this level.

Further investigations concerning the related function(s) of AcSDKP and ACE might help, in the future, to decipher the physiological role of this ubiquitous peptide, which might play an important role in the regulation of cellular hemostasis in living organisms, interalia.

Acknowledgements

This work was supported by the Ligue Nationale contre le Cancer. We thank Dr E. Deschamps de Paillette of Henry Beaufour Institute for her constant encouragement.

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(*Received in U.S.A.* 9 *January* 1996; *accepted* 26 *April* 1996)