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New properties of murine Angiotensin I-converting Enzyme (mACE) and its catalytic domains

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Table of Contents

Neue Eigenschaften des murinen Angiotensin I-converting Enzyms (mACE) und seiner katalytischen Domänen

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Zusammenfassung

Das Angiotensin-converting Enzyme (ACE) (EC 3.4.15.1, eine Metallopeptidase der M-2 Familie) ist eine typische Dipeptidyl-carboxypeptidase. Über eine derartige hydrolytische Spaltung baut ACE physiologisch wichtige Peptide wie Angiotensin-I (AI) und Bradykinin (BK) ab, und spielt folglich eine entscheidende Rolle der Herz-Kreislauf Regulation. Neben diesen gut beschriebenen Prozessen besitzt ACE einige ungewöhnliche Funktionen deren Mechanismus noch weitgehend ungeklärt ist; dazu gehören **(i)** die völlig unerwarteten endopeptidolytischen Aktivitäten des ACE bei einigen Substraten, z.B. beim Abbau von Amyloid-beta (Aβ) Peptiden; **(ii)** die Wirkungen des Spermien-ständigen ACE beim Spermien-Eizell-Kontakt, die völlig unabhängig von der Peptidasewirkung des ACE sind, und **(iii)** die rezeptorartigen Mechanismen von membranständigem ACE bei intakten Zellen nach Reaktion mit Substraten und Inhibitoren des Enzyms.

In der vorliegenden Arbeit wurde versucht, unter Nutzung von stabil-transfiziertem murinem ACE (mACE) in Modellzellen (CHO und MDCK) zur Klärung dieser Prozesse beizutragen. Außerdem wurde eine besondere Aufmerksamkeit auf die beiden Domänen-selektiven Formen den ACE gerichtet.

(a) Es wurde der langsame Abbau von Aβ-Peptiden durch mACE und seine Domänen untersucht. Überraschenderweise zeigte sich, dass beide katalytischen Domänen einen nahezu gleichartigen Abbau der Aβ-Peptide bewirken. Die in der Literatur beschriebene endopeptidolytische Wirkung von hACE auf Aβ-Peptide ließ sich auch beim mACE und bei seinen beiden katalytischen Domänen nachweisen; dabei wurden neue Spaltstellen innerhalb des Aβ-Moleküls aufgefunden.

(b) Es wurde nachgewiesen dass mACE mit Carboxypeptidase M (CPM), einem GPI-verankerten Membranprotein, interagiert. Dies gilt sowohl für lösliches als auch für membranständiges ACE und ist völlig unabhängig von der katalytischen Aktivität der Domänen des ACE. Derartige Wirkungen des ACE beim Prozess des Spermien-Eizell-Kontakts auf der Zona Pellucida wurden in der Literatur zuletzt sehr kontrovers diskutiert. Diese Ergebnisse belegen, dass bei Modellzellen die räumliche Nähe von ACE und dem GPI-verankerten CPM zu einer Interaktion und letztlich auch zu einem Release des CPM führen kann.

(c) Schließlich wurde unter Nutzung von mACE-transfizierten CHO-Zellen und nach Reaktion mit typischen ACE-Substraten oder mit ACE-Inhibitoren ein intrazelluläres Signalling (via JNK und cJun) nachgewiesen. Die Daten zeigen, dass die beiden

katalytischen Domänen in diesem Prozess nicht unabhängig von einander agieren, sondern sich im Sinne einer negativen Kooperativität beeinflussen. Die Interaktion mit Inhibitoren und Substraten führt letztlich zu signifikanten Änderungen der Expression von COX-2 und ACE.

Die vorgelegten Ergebnisse zeigen, dass das ACE offensichtlich Funktionen besitzt, die weit über den Rahmen einer typischen Dipeptidyl-carboxypeptidase-Wirkung hinausgehen. Berücksichtigt man, dass das ACE eines der wichtigsten Targets in der modernen Medizin ist, sollte die Erforschung dieser untypischen Wirkungen größte Aufmerksamkeit erlangen.

New properties of murine angiotensin I-converting enzyme (mACE) and its catalytic domains

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Abstract:

Angiotensin-converting enzyme (ACE; EC 3.4.15.1, a metallopeptidase of the M-2 family) is known as most typical dipeptidyl-carboxypeptidase. Via this mechanism, ACE degrades important peptides like angiotensin-I (Ang I) and bradykinin (BK), and plays consequently a crucial role in several circulatory processes. Besides these well described processes, ACE evinces some unusual, not fully clarified functions. Among these are (i) unexpected endopeptidolytic activities of ACE on a few substrates (ii), receptor-like mechanisms (outside-in actions) of cell-bound ACE, and (iii) the molecular mechanisms of the sperm-bound ACE in the processes of fertilization and sperm-egg-contact,

Using self-produced domain-selective forms of murine ACE (mACE), the experiments and data presented here should be regarded as contributing to the elucidation of such processes:

(a) Studying the slow degradation of amyloid-beta peptides (Aβ), it has been demonstrated that unexpectedly both catalytic domains of mACE have similar hydrolytic activity on the N-terminal part of Aβ. Moreover, the results confirm that mACE has unequivocal endopeptidolytic activities.

(b) Moreover, it is described here that ACE has GPI-targeted properties. This process is completely independent of its known peptidase activities. Such activity has been controversially discussed with respect to the essential function of ACE in the process of fertilization. The results presented here indicate that a spatial proximity between membrane-bound mACE and the endogenous, GPI-anchored CPM enables an ACE evoked release of CPM.

(c) Finally, using mACE-transfected CHO-cells, the intracellular signaling after interaction with typical ACE-substrates or with inhibitors in living cells was analyzed. The data suggest that the two catalytic domains of mACE do not function independently, which means that the signal transduction in the mACE is influenced by negative cooperatives of the two catalytic domains.

After all, these effects characterize ACE as an enzyme with multiple facets, far from being a simple dipeptidyl-carboxypeptidase. Bearing in mind that ACE is one of the most commonly used targets in modern medicine, further exploration of ACE should be more in the focus of research and will surely yield exiting news.

Key Words: ***Angiotensin-converting enzyme (ACE), catalytic domains of ACE, amyloid-beta peptides (Aβ), glycosylphosphatidylinositol (GPI)-anchored proteins, JNK activity***

List of abbreviations (alphabetic order)

ACE	angiotensin-converting enzyme
Aβ	beta amyloid peptide
Ang I	Angiotensin I
Ang II	Angiotensin II
BK	Bradykinin
BPP	Bradykinin-potentiating peptides
CHO	Chinese hamster ovary
CK2	Protein kinase CK2
CLSM	Confocal Laser-Scanning Microscopy
CPM	Carboxypeptidase M
DRB	5,6-Dichloro-ribifuranosylbenzimidazole
ECE	Endothelin-converting enzyme
FITC	Fluoresceinisothiocyanate
GPI	Glycosylphosphatidylinositol
hACE	human angiotensin-converting enzyme
hAβ	human beta amyloid peptide
IDE	Insulin-degrading enzyme
mACE	murine angiotensin-converting enzyme
mAβ	murine beta amyloid Peptide
MALDI-TOF/MS	Matrix-Assisted Laser Desorption/Ionization Time-of-flight Mass Spectrometry
MßCD	Methyl-ß-cyclodextrin
MDCK	Madin-Darby canine kidney
NEP	Neutral Endopeptidase
NSB	Non-specific Binding
PBS	Phosphate-buffered solution
PI-PLC	Phosphatidylinositol-specific phospholipase C
PVDF	Polyvinylidene fluoride
RAS	Renin-angiotensin system
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis

Introduction

Angiotensin I-converting enzyme (ACE) (EC 3.4.15.1, a metallopeptidase of the M-2 family) is the key component of the renin-angiotensin system (RAS) [1]. Somatic ACE contains two nearly identical catalytic domains which are located extracellularly [2]. ACE usually strictly acts as a dipeptidyl-carboxypeptidase, which e.g. converts angiotensin I (Ang I) to the vasoconstrictory octapeptide angiotensin II (Ang II) and inactivates the vasodilatory bradykinin (BK) into inactive peptides [3]. Consequently, the application of ACE inhibitors results in reduction of blood pressure and is an effective tool in the treatment of several cardiac diseases. Besides, this well described mechanism, ACE evinces some unusual, not fully clarified functions [4,5]. Some aspects of these unexpected functions are in the special focus of this study.

A. Degradation of Aβ-peptides by ACE. The accumulation of the so-called "Alzheimer peptide" beta amyloid peptide (Aβ) in CNS depends on a distortion of the steady state between synthesis and degradation of this peptide. The main peptidases involved in Aβ degradation/catabolism are NEP (neutral endopeptidase, EC 3.4.24.11), IDE (insulin-degrading enzyme, EC 3.4.24.56), ECE (endothelin-converting enzyme, EC 3.4.24.71) and plasmin (EC 3.4.21.7) [6]. Surprisingly, also ACE, a typical dipeptidyl-carboxypeptidase, is claimed to degrade human Aβ (hAß) and that by an endopeptidolytic attack on its N-terminal part [7]. Interestingly, Iwata et al. [8] described that NEP, the most prominent Aβ-degrading enzyme, also cleaves in this N-terminal part. In this context, should be noted that h- and mAβ differ from each other only in this N-terminal part (hAβ versus mAβ): Arg^5/Gly^5, Tyr^{10}/Phe^{10} and His^{13}/Arg^{13} [9]. This is of particular interest, as mice and rats do not develop Aβ depositions (plaques and fibrils) and any of the typical signs of Alzheimer's disease. Consequently, the aim of this studies was to compare the catabolism of m- and hAβ by ACE and its two catalytic domains using the truncated peptides Aβ(4-15). Finally it has been purposed to analyze the ACE-evoked cleaving sites in the full length Aβ-molecules [10]

B. ACE acts as a GPI-anchored protein releasing enzyme. Recently, Kondoh et al. reported that ACE is able to release glycosylphosphatidylinositol (GPI)-anchored proteins from cellular surfaces. This completely novel function of ACE is claimed to be not inhibitable by ACE inhibitors and is discussed as the long sought molecular basis for the essential function of sperm-bound ACE in male fertilization [11]. Kondoh´s paper initialized a remarkable controversy concerning this ACE-induced effect [11,12].

CHO- as well as MDCK cells are known to express carboxypeptidase M (CPM) [13,14]. Interestingly, CPM, widely distributed in mammalian tissues, belongs to the rare group of GPI-anchored peptidases [14,15]. Consequently, CHO- or MDCK-cells transfected with

mACE and consequently equipped with ACE as well as CPM, offered an ideal basis for a proof of principle to describe this most controversial ACE interaction with GPI-anchored proteins.

C. Receptor-like functions of ACE. The inhibition of ACE is a widely used therapeutic approach [16]. As a result of such chronic treatments, elevations of plasma ACE and increased expressions of ACE in different tissues have been reported [17,18]. The underlying mechanism of this inhibitor-induced ACE-upregulation is vague, but as recently postulated perhaps initiated by “receptor-like function” of ACE. Kohlstedt et al. [19,20] described that membrane-bound human ACE (hACE) - in spite of its single transmembranal domain and an extremely short cytoplasmatic tail - acts surprisingly as a “signal transduction molecule”. Moreover, they postulated that this signalling may contribute to the beneficial effects of ACE inhibitors [19]. To validate such signalling in a completely different cellular system, CHO cells were transfected with mACE or with its domain-selective forms and the catalytic functionality was proved. Thereafter, the substrate- or inhibitor-induced intracellular signalling was analyzed using different biochemical and molecular techniques.

Methods

Construction and expression of different forms of mACE: Wild type or selectively mutated murine ACE (mACE) were produced as stable transfected membrane proteins using Chinese hamster ovary cells (CHO) and Lipofectamine as transfection reagent. The principle of this technology has been described basically for hACE by Wei et al. [21]. In this process the essential zinc-binding histidines (H) in the HEMGH consensus sequences are selectively substituted by lysine (K) residues. Consequently, the following mutations inactivate the N-domain: K959,K963; and the following mutations inactivate the C-domain: K361,K365. Moreover, for negative controls for the intended biochemical and pharmacological experiments, a full-length, non-active form of mACE (K361,K365,K959,K963) was produced, containing two inactivated catalytic domains. For further details see **Sun-1** [10].

Screening of clones for ACE activity and selectivity: Enzymatic activities were measured using Hippuryl-His-Leu (degraded exclusively by the C-domain of ACE) and (Z)-Phe-His-Leu (degraded by both ACE-domains) as substrates in fluorimetric assays according to principles developed by Danilov et al. [22]. The selectivity was finally confirmed by degradation of AcSDKP (degraded exclusively by the N-domain of ACE) according to Rosseau et al. [23]. The specificity of all ACE assays was confirmed with the specific, non-peptidic ACE inhibitor Lisinopril. Further details see **Sun-1** [10].

Preparation of the cell membranes: Confluent cells were harvested, washed, centrifuged, homogenized by a glass-teflon potter, ultrasonicated and washed again as described in detail by **Sun-1** [10].

Degradation Studies of Aβ: Aβ(4-15) and Aβ(1-40), synthesized by M. Beyermann (FMP), were dissolved in water or dimethyl sulfoxide (DMSO) to form stock solutions. The different forms of ACE were incubated with 10 µM Aβ(4-15) and the reaction products were analyzed by reverse phase HPLC (C18 column). The recombinant h- or mACE was incubated with 10 µM Aβ(1-40) at 37°C overnight and the reaction products were purified by ZipTip (Millipore Corporation, USA) according to the manufacturer's protocol. For details see **Sun-1** [10].

Mass Spectrometry: The HPLC eluates and the eluates from ZipTip were collected. MALDI-TOF mass spectrometry acquisition was performed on a Voyager STR mass spectrometer (Applied Biosystems, USA) set to reflection mode. Monoisotopic peptide masses were searched against the theoretical peptide masses in the Swiss-Prot and TrEMBL protein databases. The MALDI-MS spectra were calibrated using several peaks as external

standards. For details see **Sun-1** [10]. LC/MS/MS was carried out as described in detail by Körbel et al. [24].

JNK Assay: To assess ACE-associated JNK activity, the SAPK/JNK Assay Kit (New England Biolabs) was used. In brief, the cells were serum starved for 18 hours and then treated with fresh medium containing the different compounds (10^{-7}M). The control cells were only treated with the solvent. JNK was immunoprecipitated from the whole cell lysates with aliquots of recombinant c-Jun fusion protein beads as described in detail in **Sun-3** [25].

Kinase assay: Washed immunoprecipitates were incubated with 200 µM ATP for 30 minutes. The reactions were stopped with SDS sample buffer and heated at 95°C for 5 minutes. The probes were then separated by SDS-PAGE (12%) and immunoblotted with phosphor-c-Jun (Ser63) antibody [for details see **Sun-3** (25)].

Immunoblotting: Cells were lysed in 1×SDS lysis buffer containing a protease inhibitor cocktail (Roche, Mannheim, Germany). Immunoblotting was done with respective antibody as described in detail by **Sun-3** [25].

Quantitative Real-Time PCR: Total cellular RNA was isolated using the TRIzol reagent (Life Technologies, USA). First strand cDNA was prepared by reverse transcription using the Superscript II kit (Life Technologies). Oligonucleotide probes were labelled with FAM (6-carboxy-fluorescent reporter dye) at the 5' end and TAMRA (6-carboxy-tetramethylrhodomine quencher dye) at the 3' end. Amplification was carried out for 1 cycle at 95°C for 4 minutes, 30 cycles at 95°C for 15 seconds, 60°C for 1 minute, and 68°C for 1 minute. The PCR reactions were performed in an ABI 7900 instrument using standard conditions. RNA levels of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) were used as an internal control **Sun-3** [25].

CPM assay: The reaction was carried out with Dansyl-Ala-Arg-OH as substrate in Hepes buffer, pH 7.5, at 37°C and was stopped with sodium citrate, pH 3.1. After extraction with chloroform the fluorescence was measured in the organic phase at 340-nm (excitation) and 495-nm (emission) as described by Deddish et al. [15]. For details see also **Sun-2** [26]

Immunocytochemistry (CLSM): CHO cells and mACE-transfected CHO cells were cultivated on glass coverslips and fixed in paraformaldehyde. The coverslips were blocked with goat serum in PBS for 30 minutes. CPM was detected with primary polyclonal antibody overnight. After Incubation with a FITC-conjugated goat anti-rabbit secondary antibody, the cover slips were sealed with the mounting media. The reaction was visualized using a confocal microscope system (LSM510 META, Germany). For details see **Sun-2** [26].

Release of CPM by recombinant human ACE: Confluent cells were pretreated with 10 mM methyl-β-cyclodextrin (MβCD) for 1h at 37°C. Cells (1×10^6 cells/vial) were incubated with increasing amounts of recombinant hACE or PBS alone for 2h at 37°C and then centrifuged at 300 g, 10 min. CPM activity was measured in the supernatant as described above. For details see **Sun-2** [26].

Binding assays: The cells were incubated with [^{125}I](Sar-1,Ile-8)AngII (50pM) in the presence (non-specific binding, NSB) or absence of unlabelled AngII (1μM). AT1-transfected CHO cells and non-transfected CHO were set up as control. For details see **Sun-2** [26].

Immunogold electron microscopy: MDCK cells or MDCK cells stably transfected with wildtype mACE were grown on culture dishes, fixed and incubated with CPM monoclonal antibody or ACE polyclonal antibody. Then preparations were incubated with secondary antibodies (goat anti mouse secondary antibody coupled to 10 nm gold particles and goat anti rabbit antibody coupled to 15 nm gold particles, Aurion). The final preparations were examined by electron microscopy as described by Lorenz et al. [27].

The **protein concentration** was determined according to Bradford [28].

Results and Discussion

I. Aβ degradation studies

In order to characterize the precise role of either of the two catalytic domains of mACE, the two domain-selective ACE mutants were constructed and stably transfected into CHO cells (**Fig. 2A in Sun-1**). As expected, non-transfected CHO cells had no intrinsic ACE activity. Using Z-Phe-His-Leu as substrate, the mean value for wtmACE-cells was estimated at 5.2 nmol His-Leu/min/10^6 cells. The domain-selectivity of all isolated cell clones was characterized according to a strategy developed by Danilov et al. [22] by calculating the ratio of His-Leu formation from (Z)-Phe-His-Leu (non-domain selective) and from Hip-His-Leu (C-domain selective) (**Fig. 2C in Sun-1**). Additionally, HPLC-monitored hydrolysis of AcSDKP, which is the most specific substrate for the N-domain [23], was used to confirm the correctness of domain-selective transfections. The cell clones displaying the highest rate of Z-Phe-His-Leu degradation, indicated by vertical arrows, were selected for further experimentation. The correct molecular masses (170 kDa) of the respective gene products were confirmed in Western blotting experiments **(Fig. 2B in Sun-1**).

As mentioned above, ACE is claimed to degrade hAβ by an endopeptidolytic attack on the N-terminal part of the Aβ molecule [7]. Consequently, the aim of this work was to compare the catabolism of somewhat different m- and hAβ (**see Fig.1 in Sun-1**) by ACE and to compare thereby the action of the two catalytic domains. Because of the bad manageability of full-length Aβ peptides, it was decided to use in a first step the truncated peptides Aβ(4-15). The degradation studies of Aβ(4-15) with ACE (different cellular forms of mACE or recombinant ACE) were performed as described under Materials and Methods. The quantification of Aβ degradation (peak areas) and the isolation of resulting degradation products were performed by a HPLC-separation. Manually isolated peaks were subjected to MALDI-TOF/MS.

Summarizing these results, it was found that hAβ(4-15) was obviously the much better substrate for mACE than the corresponding mAβ(4-15) (see **Fig. 4C in Sun-1)**. Moreover, there was surprisingly no real difference in the effectiveness of the N- and C-domain-specific forms of mACE in degrading the Aβ peptides (**Fig. 5 in Sun-1).** Although, the works described here were exclusively performed with an N-terminal sequence of Aβ, this is in a clear contrast to findings of Oba et al. [29] who described that exclusively the N-domain of ACE was able to degrade hAβ.

In a second step, the corresponding cleavage sites of the ACE-evoked Aβ(4-15)-degradation were analyzed. Also here, differences were found between h- and mAβ, but no differences were detectable between the two domain-selective forms of mACE. The analysis revealed that all three forms of mACE hydrolyzed h- as well as mAβ(4-15) at Glu^{11}-Val^{12}

(Table 2 in Sun-1). Consequently, it was confirmed that ACE indeed degrades Aβ peptides via an endopeptidolytic attack as described by Oba et al. [29] and by Hu et al. [7]. Using hAβ(4-15) a second cleavage site was found (His^{13}-His^{14}); this corresponds to the typical dipeptidyl-carboxypeptidase activity for ACE. The corresponding hydrolysis between Arg^{13}-His^{14} in mAβ(4-15) was never found **(Table 1 in Sun-1)**. An Asp^{7}-Ser^{8} cleavage as described by Hu et al. [7] was never found in the experiments presented here.

Due to the reduced solubility and strong aggregation behavior of full length Aβ, the investigation of its degradation by ACE required a quite different technology. After prolonged incubations with recombinant ACE the reaction products were purified by ZipTip technology and the respective gradient eluates were analyzed by MALDI-TOF/MS. The results are summarized in the Tables 3 and 4. As described above for the truncated Aβ, ACE degrades the full-length molecules endopeptidolytically (here uniformly in the positions Leu^{34}-Met^{35}; Lys^{28}-Gly^{29}; and Ser^{26}-Asn^{27}). On the other hand, it can not be fully ruled out that some of the listed cleavage sites in **Figure 6B** and **Tables 3 and 4 (in Sun-1)** in the end are the result of consecutive endopeptidase and subsequent dipeptidase activities. Again, an Asp^{7}-Ser^{8} cleavage as described by Hu et al. [7] was never found in our experiments.

II. Interactions of ACE with GPI anchored proteins

As mentioned above, ACE is most controversially discussed to have, besides its prominent function as a dipeptidyl-carboxypeptidase, also GPI-anchored protein releasing properties (GPI-ase) [11,12]. According to Kondoh et al. [11], such process should be the long term-searched molecular basis for the essential function of testicular ACE in male fertility.

Among peptidases GPI-anchored proteins are very rare, and CPM is known so far as the only GPI-anchored carboxypeptidase. The frequently used cell lines MDCK and CHO are known to express endogenous CPM. With this study we wanted to find out whether there are interactions between ACE and this GPI-anchored protein. Consequently, the mACE transfected CHO or MDCK cells provide an excellent opportunity to test the findings of Kondoh et al. [11] in a completely different cellular system.

Numerous experiments were performed to describe ACE-CPM interactions and its influences on CPM-activity. As shown in Western blots (**Fig. 1C in Sun-2**), CPM is detectable in non-transfected CHO- and MDCK cells as well as in the corresponding mACE transfected cells. The identified bands at 62 or 54 kDa correspond to the species-specific molecular masses of CPM in CHO [26] and MDCK cells [15]. The same blots were reprobed with an ACE antibody and revealed the expected bands of 170 kDa (**Fig. 1C, panel b and d, in Sun-2**). Using non-transfected CHO cells, the expected release of CPM was found after treatment with bacterial PI-PLC, but also a shedding of CPM with increasing amounts of rc

hACE (**Fig. 2A/2B in Sun-2**).

In immunochemical studies using confocal Laser-Scanning microscopy (CLSM), it was confirmed that CHO cells display high levels of membranal CPM (**Fig. 3 in Sun-2**). Moreover, a comparative quantitative analysis of fluorescence signals (transfected- versus non-transfected cells) revealed that mACE caused a small but significant reduction of CPM (FI, 65.85 versus 59.76; $p<0.001$). This small but significant reduction of membrane bound CPM in mACE transfected cells and a corresponding increase of CPM in the supernatant was confirmed with different techniques.

For further verification of these ACE-CPM interactions, chemical cross-linking studies were performed with MDCK cells and mACE transfected MDCK cells. The proximity of the two proteins was unequivocally identified by Western analysis after cross-linking (**Fig. 5A in Sun-2**). For further demonstration of direct interaction between CPM and the co-expressed mACE, we performed immunostaining studies with differently sized gold-labeled antibodies and visualized it with electron microscopy **(Fig. 4 in Sun-2).** The direct vicinity of the different gold particles reflects ACE and CPM interactions. Non of the tested ACE-inhibitors had any influence on the ACE-CPM interaction. Consequently, we have to state that the catalytic centers of ACE which cause the dipeptidylpeptidase function are not involved in the ACE-evoked “GPI-directed” action.

Summarizing the results of this chapter, it was doubtlessly confirmed that transmembranal ACE interacts with co-localized CPM, a known GPI-anchored protein. In spite of manifold experiments, the exact molecular mechanism underlying the ACE-CPM interactions and the distinct cleavage site within the GPI-CPM binding are unknown. Future studies are necessary to clarify the underlying mechanism of this GPI-targeting action. Finally, it has to be underlined that this study was not undertaken to verify or to rebut Kondoh´s theory about ACE as a sperm-bound GPI-ase [11], or even its dramatic function in the process of mammalian fertilization. But reference should again be made to the fact that it has doubtlessly found an ACE function on a GPI-anchored protein.

III. Cell signaling experiments

Some actions of ACE which apparently completely independent of the typical hydrolytic actions of this enzyme and belong consequently to the most unexpected properties of ACE. Cell-bound hACE has been previously reported to initiate a “receptor-like” signal transduction after reaction with inhibitors or substrates. In this process, JNK activation and c-Jun accumulation play an important role [19]. To investigate the relevance of domain-selectivity within the “ACE-signalling”, the different mACE-transfected cells (wt, C- or N-domain) were treated with several RAS-related compounds and inhibitors, such as AcSDKP,

Ang I, Ang II, Ang-(1-7), Bradykinin, BPP, Des-Leu^{8}-Arg^{9}-BK, Ramiprilat and Lisinopril. The JNK activity was then examined. A comparative quantification for all three forms of CHO-transfected mACE is shown in **Fig. 2A-C (in Sun-3)**. Generally, increased JNK activities were observed after treatment with ACE inhibitors or ACE substrates (**Figs. 2A and 2B in Sun-3**). Interestingly, CHO-cells transfected with the active C-domain of mACE (C-mACE) display the most intensive up-regulation of JNK-activity. On the other hand, CHO-cells with wtmACE display the weakest increase of JNK-activity. Comparing the different agents, the strongest elevations were found after treatment with prominent substrates like AngI, BK or potent ACE inhibitors.

Further JNK-activations are summarized in **Fig. 2C in Sun-3**: e.g. actions of domain-selective ACE substrates (AcSDKP), of poor substrates like (Des-[Arg^{9}]-Leu^{8}-BK) and of Ang-(1-7). Surprisingly, even AngII which is not a substrate, but the most prominent product of ACE degradation, initiates JNK activation. The interaction of ACE with its own degradation product was confirmed in binding assays. In these experiments, a weak but unequivocal binding of [^{125}I]-labelled AngII to these three forms of mACE-transfected cells was found (**Fig. 3B in Sun-3**).

Previous studies claim that a CK2-mediated phosphorylation of a Ser molecule in the short cytoplasmic tail of ACE (position Ser^{1270} in hACE) is the initial step of the “receptor-like” signaling cascade [19,30]. Own experiments have been executed to examine whether phosphorylation also plays a role in this mACE-mediated signal pathway. Actually, the enhancement of JNK-activity induced by substrates or inhibitors could be totally abolished after preincubation with DRB, an inhibitor of CK2 (**Fig. 4A in Sun-3**). For the N- or C-domain of mACE, the cell lysates were directly resolved in SDS-PAGE, immobilized to PVDF membrane and immunostained with phospho-JNK(Thr183/Tyr185). Again, the activation of JNK was completely prevented after pretreatment with DRB (**Figs. 4B and C in Sun-3**).

Some publications reported that activation of JNK by ACE inhibitor finally results in the translocation of c-Jun to the nucleus, and produces an activation of the transcription of some ACE-associated proteins [19,31-33]. Our own studies compared corresponding properties of our mACE transfected CHO-cells with endogenously expressed ACE in HUVEC-cells and could show significant, time dependent alterations of the COX-2 transcripts after addition of substrates or inhibitors (**Fig. 5A/5B in Sun-3).** Finally, an alteration of the ACE activity in mACE expressing cells was measured following signaling induction with substrates and inhibitors (**Fig. 5C in Sun-3**). Exposure of mACE transfected cells to Ang I, BK or BPB significantly increased ACE activity. Pretreatment of CHO-cells expressing mACE with SP600125, a pharmacological inhibitor of the JNK pathway [34], fully blocked the enhancement of ACE activity. This confirms again a role of JNK in ACE activation.

Interestingly, an analysis of the ACE-associated JNK-activity in the three forms of mACE does not indicate any cumulative effects of the two catalytic centers. The interaction of substrates or inhibitors with a single catalytic domain is obviously already sufficient to induce the full signalling effect. This is a new form of so-called “negative-interactions”. The term was coined by Binesvki et al. [35] to describe the interactions of the two active sites of bovine ACE in peptide degradation. Analogously, in the signalling experiments presented here, the two catalytic centers within the same molecule do not function independently and achieve in the end a “negative co-operativity”.

A final view on the Ang II-induced signalling: AngII is not a substrate of ACE, but our binding experiments confirm that Ang II binds to ACE. Therefore, we postulate that in the process of ACE-mediated signal transduction, the binding of compounds to transmembranal ACE plays an important role. Most surprisingly, our data also show that ACE activity is elevated by compounds targeting mACE in CHO cells. The molecular background of this regulatory process is not clear. Possibly, this phenomenon might originate from JNK-related effects on ACE on a post-translational level.

The aim of this work was a more detailed description of very unusual and most controversially discussed properties of ACE. Stably transfected, membrane-bound mACE and its selectively expressed two catalytic domains was used as the model in all experiments. The results doubtlessly confirm that ACE can act as an endopeptidase and moreover, that ACE is able to induce in a receptor-like manner signal transduction effects. Quite surprisingly, there are no hints on a domain-selectivity in these functions; although, in the both cases the molecular architecture of the enzyme made corresponding differences expectable. Finally, for the first time in non-germinative cells evidences were found for a GPI-hydrolyzing activity of ACE. Moreover, this GPI-cleavage is not executed by the known catalytic domains of the enzyme. Alltogether, the effects presented here characterize ACE, which is one of the most frequently described proteins and effectively used drug targets, as an enzyme/protein with very multiple facets. ACE is far from being a simple dipeptidyl-carboxypeptidase. Further exploration of the enzyme EC3.4.15.1 will surely yield exciting news. As Urs Eriksson said, its research seems to be “just in the beginning” [36].

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List of Publications:

First author publications:

Sun X, Wiesner B, Lorenz D, Papsdorf G, Pankow K, Wang P, Dietrich N, Siems WE, Maul B. Interaction of angiotensin-converting enzyme (ACE) with membrane-bound carboxypeptidase M (CPM) - a new function of ACE. Biol. Chem. 2008, **389**:1477-85.

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Co-author publications:

Pankow K, Wang Y, Gembardt F, Krause E, **Sun X**, Krause G, Schultheiss HP, Siems WE, Walther T. Successive action of meprin A and neprilysin catabolizes B-type natriuretic peptide. Circ. Res. 2007, **101**:875-82.

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Wang E., **Sun X**., et al., Biochem. Biophys. Res. Commun. 2003, **302**: 469-75.

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