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Evidence that taurine modulates osmoregulation by modification of osmolarity sensor protein ENVZ – expression

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Summary. Although the involvement of taurine in osmoregulation is well-documented and widely accepted, no detailed mechanism for this function has been reported so far.

We used subtractive hybridization to study mRNA steady state levels of genes up- or downregulated by taurine. Rats were fed taurine 100 mg/kg body weight per day for a period of three days and hearts (total ventricular tissue) of experimental animals and controls were pooled and used for mRNA extraction. mRNAs from two groups were used for subtractive hybridization. Clones of the subtractive library were sequenced and the obtained sequences were identified by gen bank assignment.

Two clones were found to contain sequences which could be assigned to the osmolarity sensor protein envZ, showing homologies of 61 and 65%. EnvZ is an inner membrane protein in bacteria, important for osmosensing and required for porine gene regulation. It undergoes autophosphorylation and subsequently phosphorylates OmpR, which in turn binds to the porine (outer membrane protein) promoters to regulate the expression of OmpF and OmpC, major outer membrane porines.

This is the first report of an osmosensing mechanism in the mammalian system, which was described in bacteria only. Furthermore, we are assigning a tentative role for taurine in the osmoregulatory process by modifying the expression of the osmoregulatory sensor protein ENVZ.

Keywords: Amino acids – Taurine – Osmoregulation – Rat – Osmolarity sensor protein ENVZ

Introduction

The role of taurine for osmoregulation from microorganisms to the animal kingdom including Man in several organ systems, under physiological and

pathophysiological conditions, is unequivocally accepted (Solis et al., 1988; Law, 1991; Schaffer and Azuma, 1992; Pasantes-Morale et al., 1993; Moran et al., 1994; Burg, 1995; Walz and Allen, 1987; Trachtman et al., 1988; Wade et al., 1988; Oja and Saransaari, 1996; Lehmann, 1989; Graham and Wilkinson, 1992; Harris et al., 1993; Burg, 1994; Hilgier et al., 1996). The mechanisms for the osmoregulatory process are, however, not fully elucidated yet. All cells are able to keep their volume within a very limited range using volume regulatory mechanisms involving changes in the concentration of osmolytes of which taurine may be of particular importance. Cell swelling in excitable tissues may occur as a result of depolarization or fluctuations in osmolarity. These conditions lead to taurine release. Detailed mechanisms for taurine release and the released taurine's actions remain to be elucidated. Pasantes – Morales and Schousboe (Pasantes-Morales and Schousboe, 1997) stressed this problem in a recent review citing a large body of evidence excluding the participation of the taurine high affinity carrier: Using a number of inhibitors of anion exchangers it has been demonstrated that both volume regulation and taurine release in brain cells are inhibited by these drugs, implicating an anion channel in the process. Also the Ca^{++} dependence of taurine release remains a controversial issue but recent studies suggest that the releasing process is not associated with Ca^{++} or Ca^{++} – channels, whereas the Ca^{++}calmodulin system or other second messengers may well be involved. Taurine also contributes to volume regulation after shrinkage of brain cells by increasing its intracellular concentration. This effect maybe accomplished by an upregulation of the sodium-taurine cotransporter along with reduced passive fluxes and increased endogenous synthesis.

GarciaRomeu and coworkers suggest a role for the anion exchange AE1 in cell volume regulation (GarciaRomeu et al., 1996; Motais et al., 1997): Molecular cloning and functional expression of AE1 from the trout erythrocyte shows that this anion exchanger can functiion as a channel mediating taurine fluxes. In the erythrocyte, the channel activation dependes on the conditions as the cell is swollen: when swelling is caused by an accumulation of electrolytes, the channel is not activated and regulation is mediated by potassium and chloride via a KCI cotransporter. When swelling is caused by hypotonic shock, the AE1 channel mediating taurine fluxes becomes activated allowing volume recovery by releasing both, taurine and other ions. Deleuze and coworkers propose an osmoregulatory effect and regulation of the whole body fluid-balance through modulation of vasopressin release (Deleuze et al., 1988). This mechanism would involve glycine receptors of which taurine can be regarded as a major natural agonist, in the supraoptic nucleus, the main site of magnocellular neurons representing the neuroendocrine regulatory loop of vasopressin (Hussy et al., 1997).

We used the molecular biological technique of subtractive hybridization approach to search for taurine – inducible genes which may be related to osmoregulation in the heart, which is probably the best studied organ in taurine research. Subtractive hybridization was employed to subtract mRNAs steady state levels in heart of taurine treated rats from control rats and vice versa. We found that two clones of the subtractive library contained

downregulated sequences with high homology to the osmolarity sensor protein ENVZ.

Methods

6 Sprague-Dawley rats, 12 weeks old, female, were fed orally 100 mg/kg body weight taurine (Sigma) for a period of three weeks and 6 animals served as controls (Institute of Animal Breeding, Himberg, Austria). They had free access to tap water and rat cake (Altromin^R) and were kept under day and night rhythm (Lubec et al., 1996). At the end of the feeding period they were sacrificed by neck dislocation, the total ventricular tissue was taken and snap frozen in liquid nitrogen. Subtractive hybridization was performed on pooled hearts of each of the two groups.

Subtractive hybridization protocol (Labudova and Lubec, 1998)

Rat heart pools of taurine treated and untreated rats were taken into liquid nitrogen and ground for the isolation of mRNA. Isolation of mRNA was performed using the Quick Prep Micro mRNA purification kit (Pharmacia Biotech Inc., Uppsala, Sweden, cat. 27 92 55 01). 1 microgram of mRNA from each (of the two) preparation was quality – checked by cDNA cloning kit (Gibco, Life Technologies, Eggenstein, Germany, cat. 18248-013) using the incorporation of [alpha – ^{32}P] dATP (Amersham, Buckinghamshire, UK, cat AA0004) with subsequent electrophoresis on 1% agarose followed by autoradiography. The reflection film (Dupont NEF 496) was exposed to the gel for a period of two hours at room temperature.

Construction of the substractive library: 10 micrograms each of mRNA from brain of DS and control were biotinilated by UV irradiation at 360 nm according to the instructions supplied in the subtractor kit (Invitrogen, Leek, Netherlands, cat K4320-01). 1 microgram of mRNA – pools each from the heart sample was subject to reverse transcriptase reaction (subtractor kit, Invitrogen) and the cDNA – pools were hybridized with the corresponding biotinilated mRNAs from controls. The substractive hybridization mixture was incubated with streptavidin according to the subtractor kit given above and thus the biotinilated molecules (non-induced biotinilated mRNAs and the hybrid [biotinylated mRNAs / cDNAs]) complexed. The streptavidine complexes were removed by repeated phenol-chloroform extraction and subtracted cDNAs were separated from the aquous phase by alcohol precipitation (subtractor kit).

In order to amplify and clone subtracted cDNAs, they were ligated with Not I – linkers followed by Not I – digestion. These Not I linked cDNAs were ligated to Not I site of sPORT 1 cloning vector (cDNA cloning kit, Gibco).

To enable visualization of subtracted cDNAs the cloned cDNAs were amplified using universal primers

I 5′-GTAAAACGACGGCCAGT-3′
II 5′-ACAGCTATGACCATG-3′

from multiple cloning site of the sPORT -1 vector (cDNA cloning kit, Gibco). Amplified cDNAs were analysed on 1% agarose electrophoresis.

Cloning of subtracted Not I – linked cDNAs

Not I linked cDNAs ligated with sPORT 1 vector were used for the transformation of highly competent INFalpha F′ E. coli cells (Invitrogen, Leek, Netherlands, cat C2020-03) and isolated plasmids were analysed by plasmid isolation kit (Quiagen, Hilden, Germany, cat 12245) and digestion with Eco RI / Hind III. Recombinant clones were sequenced by K. Granderath, MWG – Biotech (Ebersberg, Germany). Homologies were determined by computer assisted comparison of data from the genebank sequence library: fastA@ebi.ac.uk (GBALL, Gen Bank, EMBL, Heidelberg, Germany). Subtractive hybridization was performed cross-wise i.e. heart sample mRNA subtraction

from taurine treated and vice versa at the 1:3 level (mRNA taurine trteated: control mRNA).

Results

The clone P4 from the subtractive library showed high homology of 65.6% to the ENVZ osmoregulator from salmonella typhimurium. The alignment of the clone with gene bank data are shown in Fig. 1.

The alignment of clone P7 showing homology of 61.1% with the ENVZ sequence from S.typhimurium is listed in Fig. 2.

Both sequences were downregulated at least threefold, as the level of mRNAs used for subtraction was 1:3.

Discussion

Among the about 20 clones of the subtractive library, two clones showed sequences highly homologous to the osmolarity sensor protein ENVZ.

ENVZ was described so far in bacteria only (Forst and Roberts, 1994; Ishige et al., 1994; Liljestrom et al., 1988) and is a membrane located osmosensor and a typical member of histidine kinases involved in His-Asp phosphotransfer signaling (Delgado et al., 1993; Yaku and Mizuno, 1997). ENVZ is an inner membrane protein in Escherichia coli, important for sensing and required for porine gene regulation (Forst et al., 1989; Kenney, 1997). ENVZ is regulated by intracellular ATP and undergoes autophosphorylation (Tabatabai and Forst, 1997) subsequently phosphorylating OmpR (Huang et al., 1997), which then in turn binds to the porine promoters to regulate their expression. The ENVZ-OmpR phosphorelay system modulates the expression of the OmpF and OmpC major outer membrane porines (Egger et al., 1997). Forst and coworkers have shown that inactivation of ENVZZ in Xenorhabdus nematophilus affected the production of several outer membrane proteins (Forst and Tabatabai, 1997).

ENVZ is composed of a periplasmic sensor domain, transmembrane domains and a cytoplasmic signaling domain (Park and Inouye, 1997). Three enzymatic activities are associated with ENVZ: autokinase, OmpR kinase and OmpR-phosphate-phosphatase (Hsing and Silhavy, 1997). The osmotic signal is proposed to regulate the ratio of the kinase to the phosphatase activities of ENVZ to modulate the level of OmpR phosphorylation thus representing a reverse phosphotransfer (Dutta and Inouye, 1996); autophosphorylation of ENVZ occurs through a transphosphorylation reaction between two ENVZ molecules (Hidaka et al., 1997).

No comparable system has been described for the animal kingdom including Man and this is the first report of an ENVZ like – osmosensing mRNA in the mammalian system. Our preliminary results also show that this system may be regulated / modulated by taurine, although we used a pharmacological dose of this well-documented osmoregulator and we therefore cannot draw a final conclusion about an osmosensing role of taurine under physiological

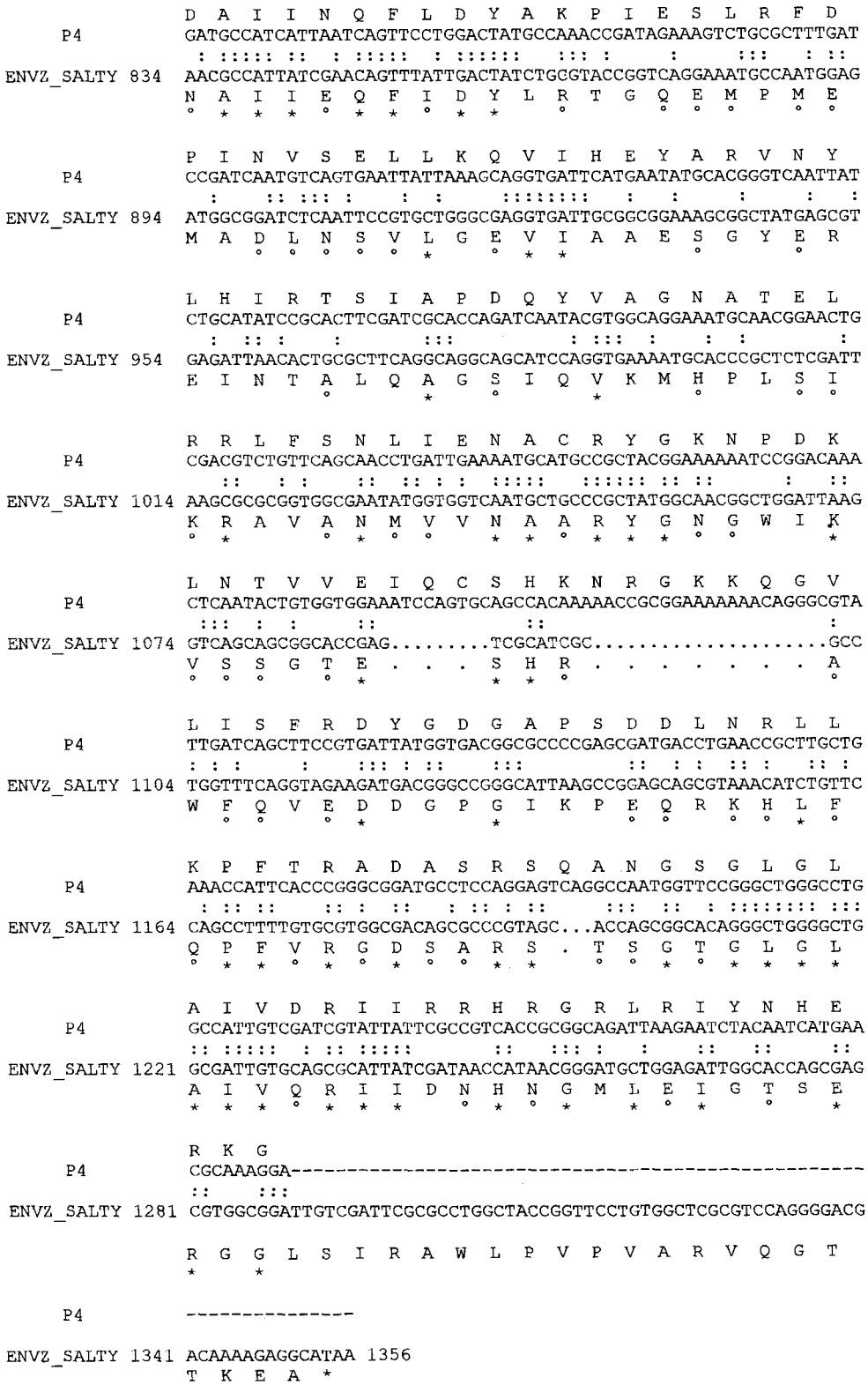

Fig. 1. The alignment of clone P4 from the taurine-subtractive library is aligned to the sequence from gene bank EMBL.: represents identical base pairs, * stands for identical amino acids and ° represents amino acids with similar function

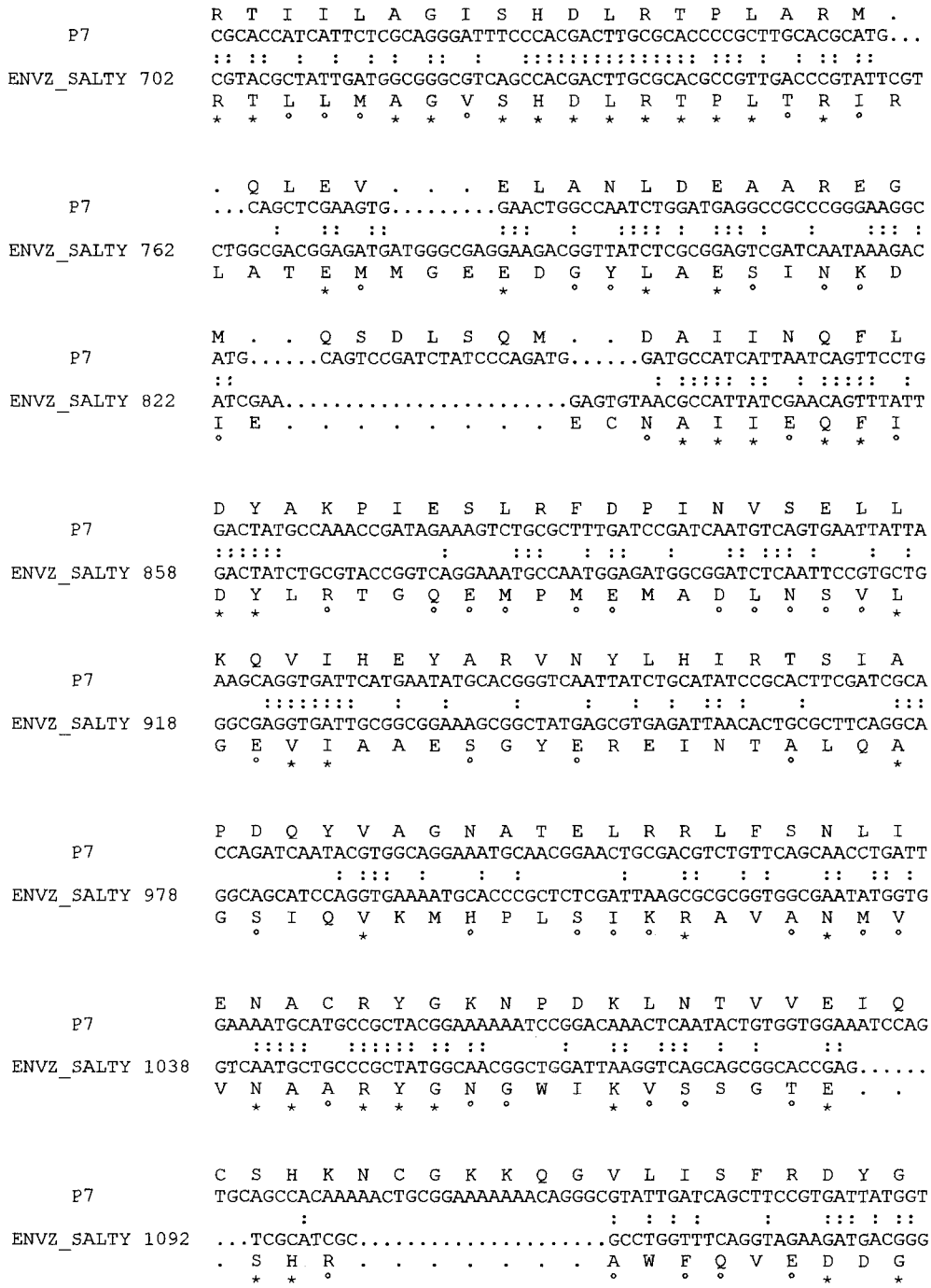

Fig. 2. The alignment of clone P7 from taurine-subtractive library is aligned to the sequence from gene bank EMBL.: represents identical base pairs, * stands for identical amino acids and ^{o} represents amino acids with similar function

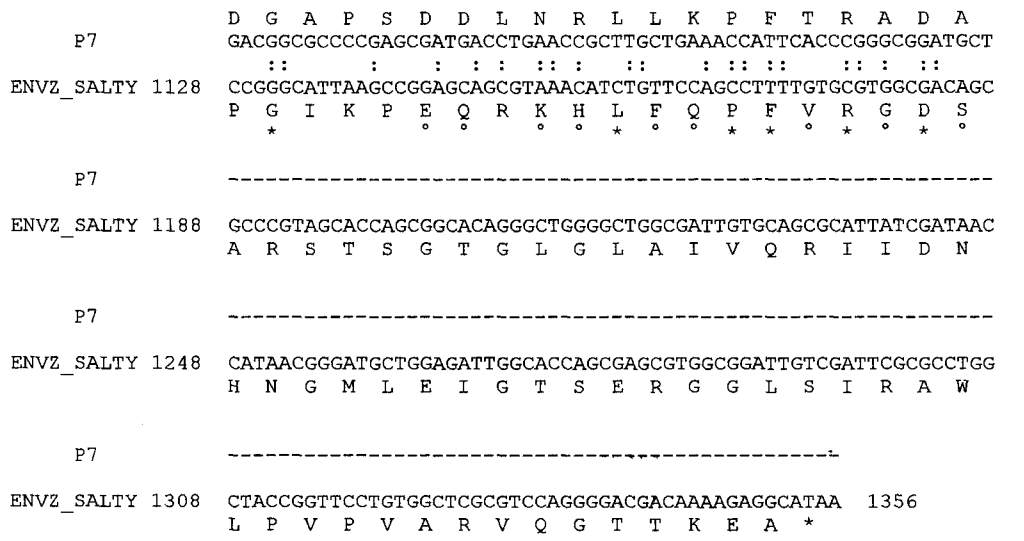

Fig. 2. *Continued*

conditions. It is, however, intriguing and pointing to a specific role, that this osmolytic compound was modifying an osmoregulatory sensor out of about twohundredthousand genes. The assignment to the ENVZ of more than 60% is further supported by the finding of two individual clones containing ENVZ-like sequences with high overlaps. The modification of ENVZ expression by taurine or a taurine metabolite may point to a mechanism for its osmoregulatory function and this forms the basis for and challenges relevant futural studies: We are providing full description of sequences from our two clones homologous to ENVZ in order to enable scientists working in the area to design antisense or knock-out studies. We are currently investigating transcriptional, protein levels of this gene, novel in the mammalian system, and performing knock out studies to elucidate a tentative physiological role.

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