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Metal accumulation and transport in the ovary of the lizard *Podarcis sicula*

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ABSTRACT

The molecular characterisation of a CTR protein is reported in the ovary of the lizard *Podarcis sicula*; this protein proves to be homologous with the mammalian high affinity copper transporter CTR1. Gene expression assessed by Northern blot hybridisation of RNA from growing ovarian follicles and eggs demonstrated that the transcript accumulated during the oocyte growth and reached the highest levels in ovulated eggs. Analysis of the copper content paralleled the profile of CTR1 mRNA, with a rapid increase of the metal concentration during oocyte maturation. These data suggest that the *P. sicula* CTR1 protein may function in the uptake and storage of the copper to be used during embryonic development.

KEY WORDS: Copper - Copper transporter - Oogenesis - Zinc.

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INTRODUCTION

Zinc and copper are essential elements required in many biological processes (Vallee & Falchuk, 1993; Linder & Hazegh-Azam, 1996). Zinc is required for the activity of many enzymes and DNA-binding proteins, and may modulate the activity of peptide hormones and neurotransmitter receptors (Beyersmann & Haase, 2001). Intracellular zinc has been shown to be essential in determining the composition and structure of chromatine as well as in selective gene expression controlling quantitative and qualitative aspects of transcription (Mazus *et al.*, 1984; Falchuk, 1998). As a consequence, zinc is required for cell division, whilst its deficiency is known to arrest proliferation and growth, and cause congenital malformations in offspring of zinc-deprived animals (Keen & Hurley, 1989).

As cofactor of a variety of enzymes, copper is required in biological reactions including photosynthesis and respiration, free radical defence, maturation of connective tissues, neurotransmitter biosynthesis and iron homeostasis (Linder, 1991; Pena *et al.*, 1999). In mammals, copper deficiency during embryonic development can result in numerous structural and biochemical abnormalities, probably due to the reduction in the activity of a number of cuproenzymes (Prohaska & Bailey, 1995; Kuznetsov *et al.*, 1996).

Although both zinc and copper play a critical function in mammalian growth and development, little is known on the occurrence and metabolic roles of zinc and copper in oocytes and embryos of non-mammalian vertebrates. It has been reported that in *Xenopus laevis*, the zinc content increases in oocytes undergoing maturation, whilst the oocyte copper content remains virtually unchanged during oocyte growth (Nomizu *et al.*, 1993). The full complement of both zinc and copper needed during development is already present when the oocyte growth and maturation is completed.

It is known that at the cellular level Zn and Cu in organisms phylogenetically very distant, such as yeast and mammals, are carried in the cells by specific proteins, Zinc-transporter (ZRT) (Palmiter & Findley, 1995; Zhao & Eide, 1996a, b; Huang *et al.*, 2002; Kambe *et al.*, 2002) and Copper-transporter (CTR) (Zhou & Gitschier, 1997; Labbe *et al.*, 1999; Lee *et al.*, 2000), located in the plasma membrane. The role played by the carriers is fundamental: their absence in mutated cells significantly increases the toxic effects of metals (Palmiter & Findley, 1995); in addition, ZRT- and CTR-deficient cells exhibit significant defects in metal-dependent enzyme activities (Cole *et al.*, 2000; Lee *et al.*, 2002).

In the present paper, we investigate the dynamics of metal uptake and transport in the ovarian follicles and eggs of the lizard *Podarcis sicula*, a reptile suitable as a model to study the chemical events occurring during oocyte growth and differentiation.

MATERIALS AND METHODS

Animals

Adult females of *P. sicula* were anaesthetised with ether and killed by decapitation; ovaries were dissected and ovarian follicles separated based on their size and stage of maturation. In addition, ovulated eggs prior to deposition were collected.

Metal determination

Metal content was determined by Atomic Absorption Spectrometry on the acid digested samples.

Reverse transcription of RNA, PCR and 5'-ends amplifications

Total RNA was isolated from the Trizol-extracted tissue samples according to the manufacturer's instructions. First strand cDNA was synthesised from 5 µg of total RNA, as previously described (Riggio *et al.*, 2002). The reverse transcription mixture was amplified by PCR using as specific primer an oligodeoxynucleotide (5'-GATGATGATGCA/CC/TATGACCTTCT-3') derived from the consensus motif MMMPMTFY present in mammalian CTR1. Amplification was performed with 5 units of AmpliTaq DNA polymerase (Perkin-Elmer). The 5' end of the cDNA was obtained by a RACE strategy according to the Marathon kit protocol (Clontech Laboratories).

The PCR products were subcloned using the Topo TA cloning kit (Invitrogen) and sequenced on both strands by the dideoxy method (Sanger *et al.*, 1977) using the T7 sequencing kit (Amersham Pharmacia Biotech).

cDNA expression in yeast cells

To express lizard CTR1 in yeast cells, a PCR fragment encompassing the cDNA coding region was inserted into the *S. cerevisiae* expression plasmid p413-GPD (Mumberg *et al.*, 1995).

The yeast strain used in this study was MPY17 (genotype *MATa gal1 trp1-1 his3Δ200 ura3-52 ctr1::ura3::Kn^r ctr3::TRP1 his3 lys2-801 CUP1^r*) (Pena *et al.*, 1998). Yeast cells were grown in YPD rich medium or YPEG medium in which cell growth requires functional cytochrome oxidase activity for mitochondrial respiration. Yeast transformations were performed by lithium acetate/single-stranded DNA/polyethylene glycol method (Gietz *et al.*, 1995).

Copper uptake assay in Hek293 cells

Human embryonic kidney (Hek293) cells were cultured in DMEM medium (GIBCO) with 10% foetal bovine serum under 5% CO_2. Cells were transfected with the pcDNA3.1 vector (Invitrogen) or pcDNA3.1 expressing the lizard cDNA under control of the CMV promoter. Transfections were performed using FuGENE^{TM}6 (Roche) according to the manufacturer's instructions. Twenty-four hours after transfection, cells were collected from transfected dishes and evenly divided for ^{64}Cu uptake assays. ^{64}Cu (2 µM as $CuCl_2$ in 0.1 M HCl) was added to Hek293 cell culture medium 2 days after transfection with empty vector or with the lizard CTR1 expression vector. Cells were incubated for 5 min, and then the medium was aspirated and Cu uptake was quenched by adding 10 mM ice-cold EDTA. Cells were washed 3 times with ice-cold EDTA, resuspended in 0.1% SDS / 1% Triton-X 100/ PBS buffer for lysis, and aliquots of cell lysate were counted by using a counter (Packard Cobra II). Parallel experiments were conducted at 4 °C for cell surface ^{64}Cu binding, which was subtracted from the values at 37 °C to obtain net copper uptake values. Copper uptake was calculated using a standard curve and normalised to protein concentrations of cell lysates.

Northern blot analysis of RNA

Total RNA from previtellogenic oocytes, vitellogenic oocytes and eggs was electrophoresed through a 1.2% agarose gel containing 2.2 M formaldehyde, as previously described (Riggio *et al.*, 2002). RNA was blotted on nylon membranes (Millipore), and probed for CTR1 mRNA. Membranes were prehybridised and hybridised in ULTRAhyb solution (Ambion) at 42 °C for 20 h. The hybridisation to target RNA was detected by autoradiography. The integrity and loading consistency of RNAs was assessed by rehybridation of the stripped membranes with a specific 18S rRNA probe.

RESULTS AND DISCUSSION

Metal content in oocytes and eggs

Analyses of zinc and copper contents were carried out on ovarian follicles at different stages of maturation and on eggs. The results in Fig. 1 show that lizard oocytes accumulated both metals during the preparatory phases of oogenesis, reaching the maximum concentration of metals in eggs. These data are at variance with those observed in *Xenopus* oocytes, where copper content remains constant throughout the entire process of maturation (Nomizu *et al.*, 1993). Furthermore, in most biological systems including mammals, the concentration of copper is about four or five times lower than that of zinc (Kirchgessner *et al.*, 1982; Masters *et al.*, 1983; Nomizu *et al.*, 1993), while in *P. sicula* oocytes and eggs the copper content is as high as that of zinc. Taken together, our results suggest comparable requirements for both metals during embryonic development.

Molecular cloning and functional characterisation of lizard high affinity copper transport protein (CTR1)

The observation that ovarian follicles accumulate a large amount of copper prompted us to investigate the existence of copper transport proteins in lizard. Using RT-PCR and 5'-RACE strategies, we cloned a cDNA encoding a polypeptide of 192 amino acid residues (GenBank Accession number: AJ421475), homologous with

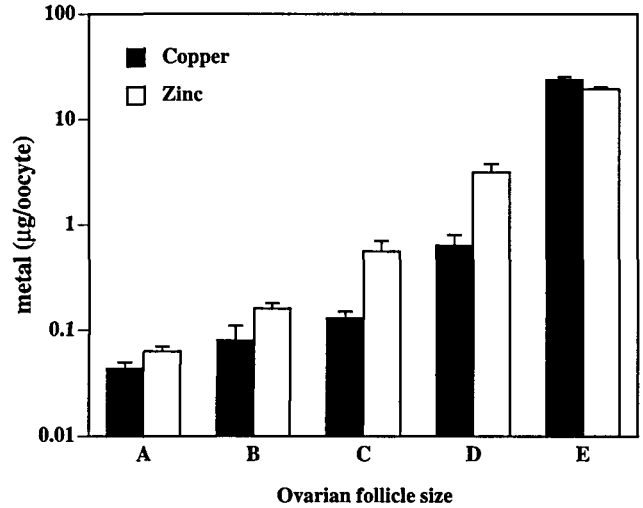

Fig. 1 - Copper and zinc content in different classes of ovarian follicles and in eggs. A, follicles less than 1 mm in diameter; B, follicles 1-2.0 mm in diameter; C, follicles 2.1-4 mm in diameter; D, follicles 4.1-9 mm in diameter; E, eggs. Values are shown in logarithmic scale.

the Cu-transporter (CTR1) from mammals (Zhou & Gitschier, 1997; Lee *et al.*, 2000). In order to verify that lizard CTR1 functions in copper acquisition, the lizard CTR1 ORF was expressed in yeast strain MPY17 defective in copper transport, that exhibits a defect in mitochondrial respiration (Labbe *et al.*, 1999). The expression of lizard CTR1 was able to cure the respiratory defect associated with the yeast strain mutation, demonstrating that lizard CTR1 interacts directly with copper and plays a role in copper transport. Moreover, the expression of lizard CTR1 protein, under the control of a strong promoter, is able to stimulate copper uptake in cultured human cells (Fig. 2), providing further support for the hypothesis that the lizard CTR1 functions in copper acquisition.

These results suggest the presence of homologous systems for copper transport in reptiles and mammals.

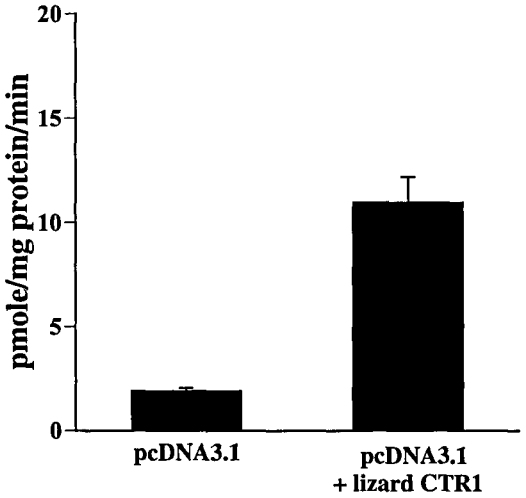

Fig. 2 - Expression of lizard CTR1 in Hek293 cells, transiently expressing lizard CTR1, which were used to measure ^{64}Cu uptake and compared with cells transfected with the empty vector.

Expression of lizard CTR1 mRNA in growing ovarian follicles

The expression levels of lizard CTR1 transcript in growing ovarian follicles and eggs were assessed by Northern blot analysis of total RNA. The data in Table I show that the amount of CTR1 mRNA increases during oocyte growth and accumulates mostly in the eggs, in parallel with the accumulation of copper.

TABLE I - *Detection of lizard CTR1 mRNA in ovarian follicles and eggs.*

Ovaria follicle diameter	Lizard CTR1 mRNA content (arbitrary units)
0.1-2	44±5
2-9	70±5
Eggs	81±5

Recent studies on CTR1 heterozygous and homozygous deletion mice demonstrated the essential role played by the mammalian copper transporter in copper homeostasis and embryonic development (Kuo *et al.*, 2001; Lee *et al.*, 2001). The present data suggest that also in the reptile *P. sicula* the CTR1 protein can be part of the copper trafficking machinery responsible for uptake and storage of the metal to be used during embryonic development.

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