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# A novel ubiquitin carboxyl terminal hydrolase is involved in toad oocyte maturation

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## ABSTRACT

p28, a 28kD protein from toad (Bufo bufo gargarizans) oocytes, was identified by using $p13^{suc1}$-agarose affinity chromatography. Sequence homology analysis of the full-length cDNA of p28(Gene Bank accession number: AF 314091) indicated that it encodes a protein containing 224 amino-acids with about 55% identities and more than 70% positives to human, rat or mouse UCH-L1, and contains homological functional domains of UCH family. Anti-p28 monoclonal antibody, on injecting into the oocytes, could inhibit the progesterone-induced resumption of meiotic division in a dose-dependent manner. The recombinant protein p28 showed similar SDS/PAGE behaviors to the native one, and promoted ubiquitin ethyl ester hydrolysis, a classical catalytic reaction for ubiquitin carboxyl terminal hydrolases (UCHs). The results in this paper reveal that a novel protein, p28, exists in the toad oocytes, is a UCH L1 homolog, was engaged in the process of progesterone-induced oocyte maturation possibly through an involvement in protein turnover and degradation.

**Key words:** *p28, cDNA clone, recombinant expression, ubiquitin carboxyl terminal hydrolase, oocyte maturation.*

## INTRODUCTION

After hibernation, the fully-grown oocytes of toad (Bufo bufo gargarizans) are physiologically arrested at the $G_2$/M border of first meiotic prophase and have acquired the competence to resume meiotic division. In response to progesterone, the oocyte undergoes germinal-vesicle breakdown (GVBD) and is arrested at MII phase[1]. The maturation-promoting factor (MPF) activity was first found in mature frog eggs [2], [3] . Our previous studies indicated that the fully-grown oocytes derived from toads reared at higher temperature (28℃) could not undergo GVBD after progesterone treatment. But once these oocytes were injected with oocyte plasma consisting of activated MPF, they were able to carry out GVBD[1]. So, MPF was shown to be a universal regulator of the phase transition from $G_2$ to M both in meiotic and mitotic cells.

MPF consists of two subunits, a catalytic subunit p34cdc2 and a regulatory subunit cyclin B[4]. The concentration of cyclin B increases steadily from late S phase up to its peak at the phase transition point from $G_2$ to M, whereas the level of $p34^{cdc2}$ remains relatively constant throughout the cell cycle[5],[6]. The modulation of cyclin concentration by synthesis and degradation is of special importance for the control of MPF (maturation/M-phase promoting factor) activity[7]. The cyclin concentration is de-

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termined by a specific equilibrium between its synthesis and degradation[8]. Cyclin B is degraded through Ub-dependent pathway. In contrast to cyclin B ubiquitination, which occurs only in mitotic extracts, the 26S proteasome, a multisubunit protease specific for multiubiquitinated substrates[9], [10], is active throughout the cell cycle, suggesting that the cyclin ubiquitination rather than its degradation, is cell cycle regulated throughout cell cycle.

$p34^{cdc2}$ has been shown to be associated with the 13 kD suc1 gene product[11]. p13 conjugated affinity columns could absorb $p34^{cdc2}$ and some other Cdk proteins, alone or in a united form with its regulatory subunit cyclins[12], [13]. Phosphorylated forms of the cyclosome from clam oocytes, one around 1, 500 kDa complex with a regulated cyclin-ubiquitin ligase activity that targets cyclin B for degradation, was reported to bind to p13SUC1[14] In this paper, using p13-agarose affinity methods, a protein of 28 kDa nominated as p28, was among the isolated proteins of lower molecular weights than those of cyclosome subunits. To seek protein factors possibly associated with cyclin B1/$p34^{cdc2}$, three proteins binding to p13 were noticed. The monoclonal antibodies against p28 were prepared, and microinjection of these antibodies into oocytes revealed that monoclonal antibody against p28 was able to abolish progesterone-induced GVBD. The p28 cDNA was screened with anti-p28 monoclonal antibody as a probe. The alignment results of the deduced animo acid sequence of p28 revealed that p28 has a homology most closely to human UCH L1. Based on Wilkinson's method[15], p28 was specified to possess a typical hydrolytic activity on UCH substrate UboEt. So we suggest that p28 is a toad UCH (nominated as tUCH) involved in the oocyte maturation.

## MATERIALS AND METHODS

### *Oocytes*

Fully-grown oocyte (about 1.8 mm in diameter) were collected from artificially hibernated toads (Bufo bufo gargarizans) caught from the countryside fields of Shandong Province. The oocytes used in the microinjection was divested manually, and the oocytes used for cDNA library and protein extracts were obtained from selected toad ovary fragments underwent a two-step digestion as Lu described[16]. To obtain oocyte protein extract, the isolated oocytes were homogenized in EB (20 m*M* Hepes pH 7.5, 80 m*M* glycerophosphate, 50 m*M* NaF, 20 m*M* EGTA, 15 m*M* $MgCl_2$, 1 m M DTT, 200 m*M* PMSF, 3 $\mu$g/ml Leupeptin), following L～u[16]. The maturation of oocytes was judged by germinal-vesicle breakdown (GVBD) after progesterone stimulation[16].

### *$p13^{SUC1}$-agarose affinity chromatography*

The oocytes were homogenized in EB, and the supernatant was removed as oocyte protein extract after a centrifugation (36,000 羚g, at 4℃ for 1 h). Following the Jessus' method[17], the oocyte protein extracts were treated at room temperature with ATP system (1 mM ATP, 80 $\mu$g creatine kinase and 10 m*M* creatine phosphate) in EB, then diluted to 1 mg/ml protein concentration. 500 ml oocyte extract (1 mg/ml total protein) was incubated for 2 h at 4ºC under constant rotation with 50 $\mu$l sepharose CL-6B (prewashed with EB). After a centrifugation (1,000×g, at 4oC for 2 min), the supernatant was removed and incubated with 20 ml p13-agarose beads (Oncogene) under constant rotation for another 6h at 4oC. p13-agarose beads were further washed three times in EB and then suspended in 1×SDS-PAGE sample buffer. The supernatant was processed for SDS-PAGE and Western blotting immediately after boiling for 5 min and a brief centrifugation [16].

### *Preparation and purification of anti-p28 monoclonal antibody*

The proteins derived from oocyte extract were separated by using 10% SDS-PAGE. The gel band, which was corresponded to p28 were excised, eluted and used for immunizing BALB/c male mice. The hybridomas secreting anti-p28 monoclonal antibody were screened through ELISA and then cloned. The anti-p28 monoclonal antibody (IgG1) was purified by using Protein A Sepharose CL-4B affinity chromatography.

### *Isolation and sequencing of p28 cDNA*

The toad oocyte cDNA library was constructed as described in Kit manuals (TimeSaver cDNA Synthesis Kit, Directional Cloning Tool Box, Lambda gt11 Sfi-Not Vector, and Ready to go Lambda Packaging Kit, all from Pharmacia Biotech.). A 0.7-kb fragment was screened with anti-p28 monoclonal antibody from the cDNA library. A full-length p28 cDNA was obtained by using the cDNA fragment as a probe from the same library. The cDNA clones were sequenced by Shanghai GeneCoere Biotechnologies Company.

### *Preparation of recombinant GST-p28*

The p28 gene coding region fragment was obtained by PCR with a Sal I restriction site added 5 primed end primer : 5' ...CG GTCGAC GCG TTG TCA CCT ATT GAG ATT AAC CC ... 3', and a Not I site affiliated 3 primed end primer: 5' ... TT GCGGCCGCTTA TGC AGC CTT GAC AAG AGC C...3' (Shanghai Sangon), from the p28 cDNA full-length sequence contained vector l gt11 DNA. The obtained p28 gene fragment was inserted orientationally into the expression vector pGEX-4T-3 (Pharmacia Biotech.), then transformed into E. coli (DE3). The construct was authenticated by sequencing with pGEX general

sequencing primers (Shanghai Genecore). The expressed GST-p28 fusion protein was mainly in the lysed supernatant and purified through glutathione Sepharose 4B column. Typically, 50 $\mu$g of GST-p28 was obtained from 1 L DE3 bacterial culture. The p28 protein moiety was removed by cleaving the GST-p28 with bovine thrombin (Pharmacia Biotech.).

### *Preparation of UCH substrate UboEt*

The classic substrate for UCH, the carboxyl-terminal ethyl ester of ubiquitin (UboEt), was synthesized by a trypsin-catalyzed transpeptidation, separated from the reaction mixture through gel-filtration and ion-exchange, and dialyzed against ultra-pure water, then stored frozen at -20℃[15].

The above-mentioned purified UboEt was characterized by high-performance liquid chromatography (HPLC) after being incubated it with 2 %(w/w) N-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin (Sigma) for 10 mins at 37℃[15].

### *Assay of UCH activity*

Following Wilkinson[15] and Piccinini[18], using UboEt as substrate, the UCH activity was assayed. In each 20 $\mu$l final volume sample, it contained 35 m*M* phosphate buffer (pH 7.2), 50 $\mu$*M* UboEt, 0.2 m*M* EDTA, 10 m*M* DTT and specified concentrations of GST-p28 product. At the given time, the reaction was stopped with perchloric acid at a final concentration of 0.2%. After centrifugation, samples were in turn loaded on Sephasil 5 $\mu$m ST 4.6/250 peptide C8 reverse-phase column (Amersham Pharmacia Biotech.) equilibrated at a flow rate of 1 ml/min with 50 m*M* $NaClO_4$ and 0.07% $HClO_4$ in 42% acetonitrile, and then subjected to isocratic HPLC analysis to assay the UCH activity.

### *Micro-injection of antibody*

Ovarian membrane and follicular surrounding oocytes were divested manually. 70 nl of purified anti-p28 monoclonal antibody at different concentrations (100-700 ng per oocyte) was injected into the nude oocytes. Equal volumes of other irrelevant monoclonal antibodies against a 24 kDa or a 26 kDa protein, derived from toad oocytes, were employed as negative controls. After injection, oocytes were incubated in Ringer's solution containing $10^{-6}$ *M* progesterone at 18℃ for 20 h. Then the oocyte maturation percentage was judged by GVBD[1].

## RESULTS

### *p28 was included in proteins absorbed by p13SUC1 -agarose beads*

Different proteins including p28 were isolated from the oocytes extract by using $p13^{SUC1}$ -agarose affinity method. Highly specific anti-p28 monoclonal antibodies were prepared by using the purified p28 as immunogen. The results obtained from Western blotting analysis revealed that p28 was included in the proteins isolated from the oocyte extract by

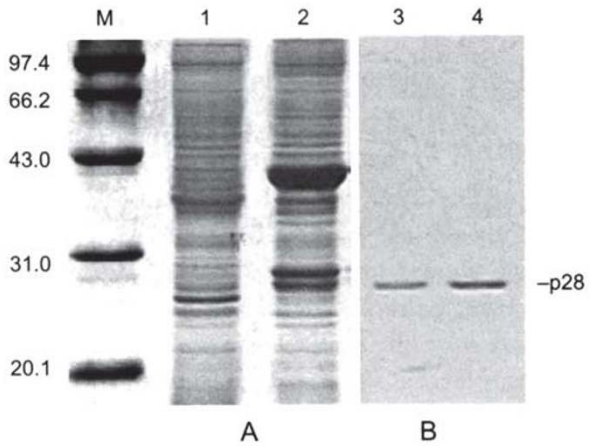



**Fig 1. p28 could be bound to p13*SUC1* agarose beads**
Lane M was loaded with low molecular protein standards (Shanghai Lizhudongfeng Biotechnique company). Lane 1 and 4 were each loaded with 20 $\mu$g of the oocyte extract; lane 2 and 3 each contained 1/2 volume of the p13SUC1 affinity isolated protein mixture from 500 $\mu$g oocyte extract. Panel (A) The protein samples were resolved by SDS/PAGE (12% gel), then stained with Coomassie brilliant blue. Panel (B) The samples were subjected to SDS/PAGE as in Panel (A), but then resolved bands were transferred to nitrocellulose membrane (Schleicher and Schuell Inc.). The blot was probed with the monoclonal antibody (200 ng/ml) against the native p28, and the immunological complex was detected with AP-labeled anti mouse IgG (1/500).

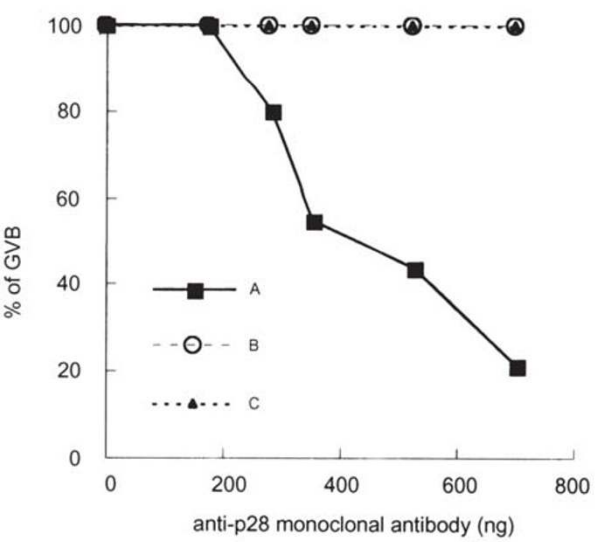



**Fig 2. The inhibitory effect of anti-p28 monoclonal antibody on maturation of ooctyes induced by progesterone stimulation**
The oocytes were micro-injected with anti-p28 monoclonal antibody (A), anti- 24kDa protein monoclonal antibody (B) and anti- 26kDa protein monoclonal antibody (C) respectively. The maturation of oocytes was judged by GVBD when it was 20 h after progesterone stimulation.

p13$^{SUC1}$ affinity absorption. For many isolated protein bands were revealed by SDS/PAGE as expected, we could not figure out whether p28 bound to p13$^{SUC1}$ directly or mediated by other proteins indirectly to p13$^{SUC1}$ (Fig 1). Certainly there is possibility that p28 can bind to p1$^{SUC1}$ through cyclin B1/p34$^{cdc2}$ or cyclosome subunits or other proteins.

*The progesterone-stimulated oocyte maturation could be inhibited by anti-p28 monoclonal antibody*

Prior to progesterone stimulation, the purified anti-p28 monoclonal antibody was injected into oocytes at different concentrations for observing the maturation percentages of oocytes, which were

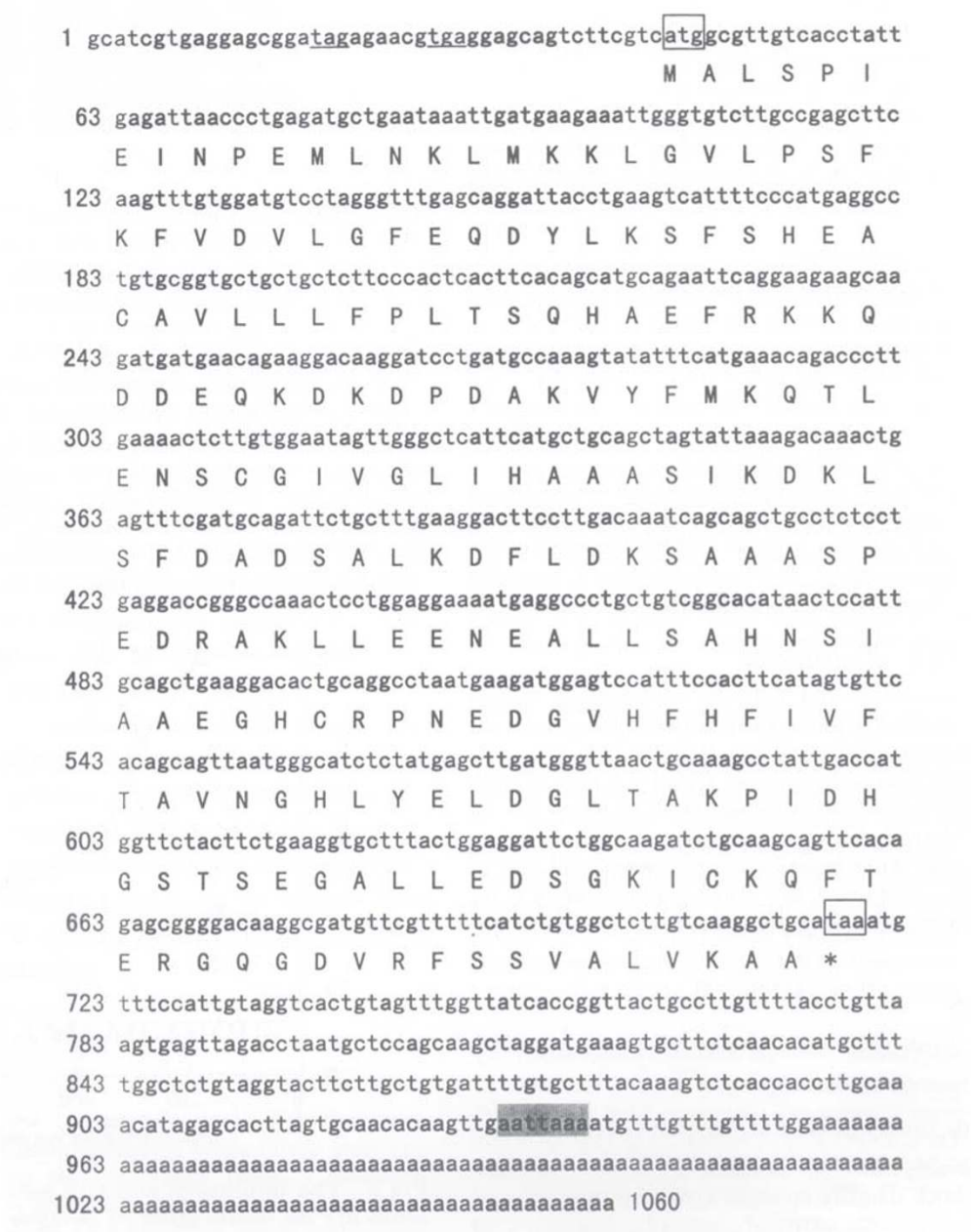



**Fig 3. Nucleotide sequence and deduced amino acid sequence of p28 cDNA**
In the nucleotide sequence, the underlined triplets indicate two stop codons lying at the 5' end untranslational region. The start and stop codons of the open reading frame are boxed, and the putative poly(A) adding signal (aattaaa) are shaded.

judged by GVBD (Fig 2). The results showed thatanti-p28 monoclonal antibody could inhibit progesterone-induced ooctye maturation in a dose-dependent manner with the maximum inhibition ratio: up to 80% at a dosage of 700 ng/70 nl. No inhibitory effect was found in both negative controls (Fig 2).

*Characteristics of p28 cDNA and its deduced amino acid sequence*

The full length of p28 cDNA sequence (Fig 3) screened from the oocyte cDNA library, which encodes a protein of 224 amino acids, consists of 1060 bp with the start codon at 45-47 base site position and the stop codon at 717-719 base site position. There is a typical consensus sequence for initiation of translation[19]. Two stop codons, TAG and TGA in turn exist in the 5' untranslational region, upstream from the start codon, at base sites 18-20 and 27-29. A tailing signal ATTAAA and a polyA tail exist at the 3' end. The open reading frame encodes a protein of 224 amino acid residues, with a deduced molecular weight of 24.4 kD, which was different from the SDS/PAGE apparent molecular weight of p28 (Fig 3).

*The prepared UboEt has peculiar HPLC peak*

The UboEt was synthesized and purified by gel-filtration and cation-exchange chromatography according to Wilkinson et al[15]. The HPLC results showed that the purity of prepared UboEt is high, and has a peculiar peak, easily distinguished from that of Ub and 74 amino acid trypsin catalyzed hydrolysis product of Ub (Fig 4). The trypsin hydrolysis mapping of UboEt was corresponded to what was introduced in Wilkinson et al[15]. So, the synthetic UboEt is appropriate for the UCH activity assay.

*p28 showed specific catalytic activity on UboEt hydolysis*

UCH L3 was shown to catalyze the hydrolysis of UboEt into Ub and ethanol. UCH L3 was used at the final concentration of 400 nM as a positive control (Fig 5(2)), and the positive reaction results assured that the UCH substrate UboEt synthesized in our laboratory is qualified, and all the UCH activity assay steps are reasonable. In comparison with GST, which results is presented in Fig 5(1), GST-p28 has an obvious catalytic action on UboEt hydrolysis, as shown in Fig 5(3) and 5(4). When the initial con-

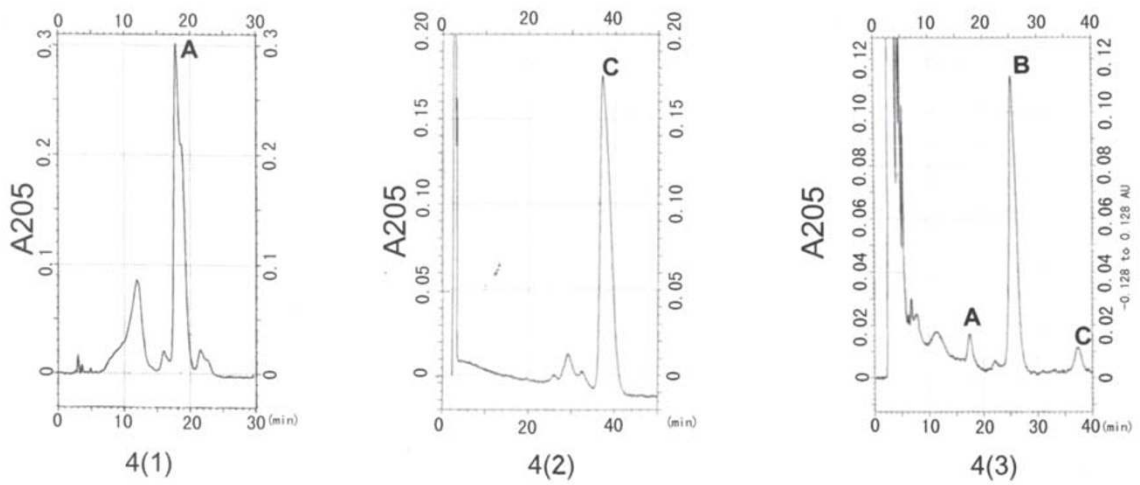
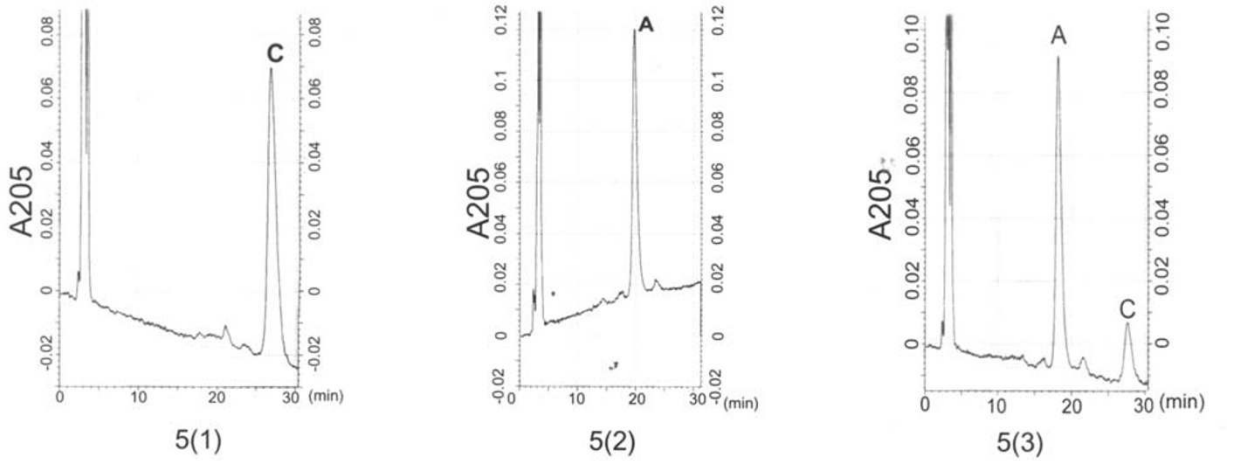



**Fig 4. The characterization of the prepared UboEt**
**(1)** The HPLC result of 50 mg commercial ubiquitin at the concentration of 1 mg/ml in ultra-pure water takes on a dominant peak indicated by A. **(2)** Peak C represents 8.5 $\mu$g of the synthesized ubiquitin ethyl ester (UboEt) in a 20 $\mu$l sample volume (50 $\mu M$). **(3)** The hydrolyzed UboEt sample, in a total volume of 20 $\mu$l, contained UboEt 5 $\mu M$, Tris-HCl (pH 7.4) 50 $\mu M$, TPCK treated Trypsin 0.5%, reacted at 37℃ for 10 mins. The reaction was stopped with perchloric acid. Most UboEt is hydrolyzed into 74 amino acid product (peak B).



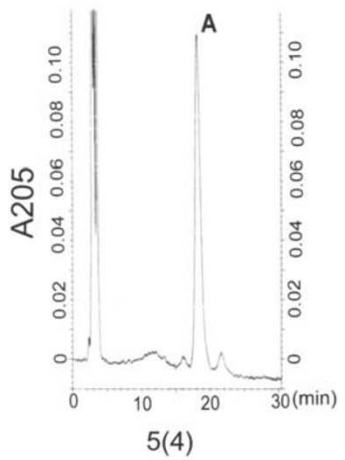




**Fig 5. The HPLC assay of UCH activity of p28**
Each 20 $\mu$l sample contained potassium phosphate (pH 7.2) 35 m*M*, EDTA 0.2 $\mu M$, dithiothreitol (DTT) 10 $\mu M$, UboEt 50 $\mu M$. In control samples (1), added GST 400n*M*; in positive control samples (2) added rabbit UCH L1 up to 400 n*M*; in others, as test samples (3) and (4), added GST-p28 to a final concentration of 400 n*M*. Kept all samples at 37℃ for a indicated time, (1) (2) and (4) 50 mins, (3) 25 mins, then the reactions were terminated by adding perchloric acid. Ub peak and UboEt peak are indicated by A and C respectively.

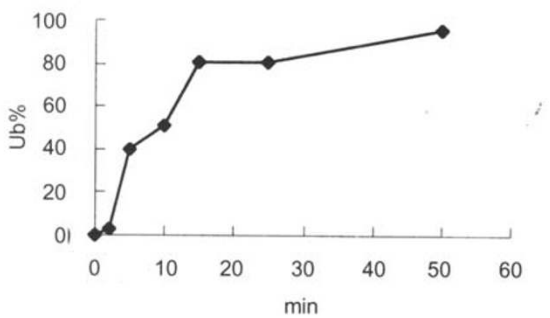



**Fig 6. A time-response curve of p28 activity**
In the reaction mixture of 200 $\mu$l, whose components were specified as in the legend of Fig 4, GST-p28 was added to a final concentration of 400 n*M*. At each time point of 0, 2, 5, 10, 15, 25, 50 mins, a 20 $\mu$l aliquot of the reaction mixture was terminated with perchloric acid, then the relative content of Ub was analyzed by HPLC to evaluate the UCH activity of p28.

centration of UboEt reaches up to 50 mM, and p28 ata final concentration of 400 nM, the catalytic reaction is weak at the beginning, then its rate rises as the time goes on till the conditions become adverse. So the time-response curve presents "S" shape, as revealed in Fig 6.

It was reported that UCH L1 (PGP 9.5) had been purified from bovine retina; its UCH activity was determined by using UboEt as substrate; and in the result the time-response reaction curves took on a linear shape when relatively low concentrations (1-8 $\mu M$) of UboEt were taken[18]. But in our situation, the higher initial concentration of UboEt inhibited the catalytic action of p28 (50 $\mu M$). As the substrate inhibition decreases with its content in the reaction mixture, the UCH activity of p28 increases. When UCH activity attains to a certain level, it turns to falling down owing to the occurrence of other unknown adverse conditions (Fig 6).

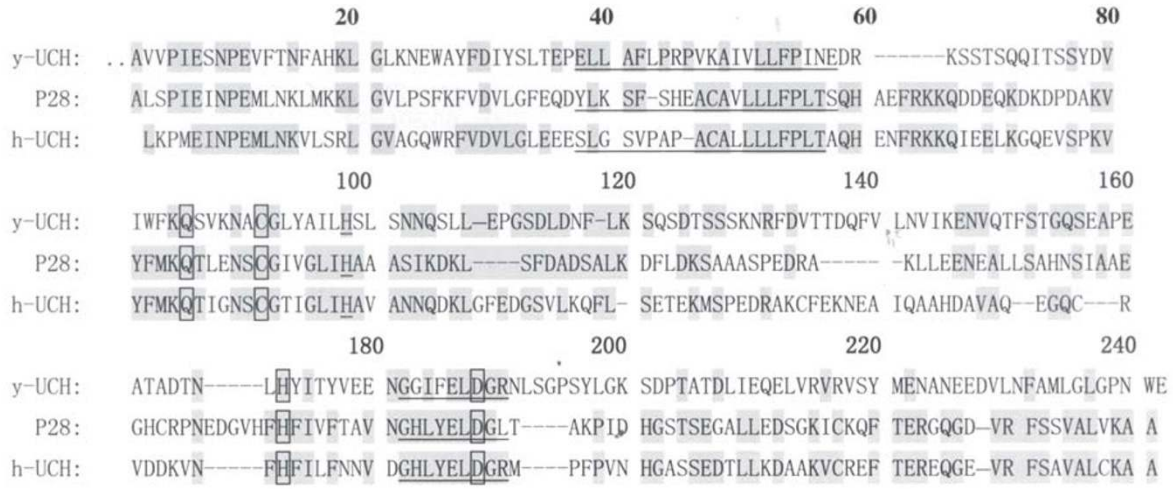



**Fig 7. Alignment of the amino acid sequence of p28 with yeast UCH (y-UCH) and Human UCH (h-UCH)** Identical amino acid residues are shaded .The residues consist of the activity center are framed. Two parts underlined are the elements joining in the ubiquitin recognition site. Database entries of the proteins aligned are: UBL1-YEAST (SW: P35127), tUCH (GB: AAG34168), and human UCH L1 (SW: P09936)

## DISCUSSION

P34$^{cdc2}$ of almost any organism have been found able to associate with yeast p13SUC1 for their conservation[13]. Recent research revealed that p13 could also absorb directly or indirectly the subunits of cyclosome[14]. Using p13-agarose affinity chromatography, we found that a 28 kD protein (p28) from fully-grown toad oocytes could be bound to p13, predicting that it might be a new candidate joining (directly or indirectly) the p34$^{cdc2}$-cyclinB complex. Purified anti-p28 antibody could effectively inhibit progesterone-induced GVBD in a dose-dependent manner as it was injected into oocytes that had acquired the competence for meiosis resumption. This result suggests that p28 is involved in the maturation process of oocytes.

Amino sequence alignments using BLAST in SwissProt database revealed that there exists a high-level sequence homology of p28 to the ubiquitin carboxyl terminal hydrolases of human and yeasts (Fig 7), about 55% identities and 70% positives. The primary structures of p28 contains the conserved regions of UCH family as the human and yeast homologues do, and the residues, Gln84, Cys90, His162 and Asp177, might be essential for catalytic activity center[20],[21] . The ubiquitin binding sites of p28 should also be highly-conserved, and the conservation of residues 37 to 56 and 170 to 180 seem to predict their possible roles in the ubiquitin recognization[22].

Although a number of UCHs have been identified from various organisms, only a few have been purified and characterized in vitro. Among the identified UCHs, it was only reported that UCH-D (Drosophila ubiquitin C-terminal hydrolase) is appeared in *D. melanogaster* oocytes[23].

Increasing evidences indicate that the UCH abnormities contribute to the pathogenesis of diseases of the human nervous system for large amounts of ubiquitin-conjugates have been found in abnormal cytoplasmic inclusions and altered axons of the nervous system, such as Alzheimer's disease[24]. As a reasonable explication of the over-expression of UCHs in tumors, UCHs might be involved in the release of ubiquitin from tagged proteins, i.e., deubiquitination, by which the degradation of cyclins decreases[25]. Certainly, we could also suppose that p28 takes part in the cyclin B1 level regulation which is involved in the G2/M transition during oocyte maturation in a similar mechanism, for p28 is shown to possess UCH activity, to have high homology to UCH L1, and to be associated with p13$^{SUC1}$, which binds with cyclin B1/p34$^{cdc2}$. However, as proteolysis through the ubiquitin-proteasome pathway may also

implicated in the degradation of some proteins that mediate the signal transduction, the involvement of tUCH in regulation of progesterone- induced signal transduction during oocytes maturation could not be excluded.

It is well known that cyclin B increases steadily from late S phase and reaches its peak at the G2 /M border. We suggest that p28, as a UCH enzyme, may ensure the accumulation of cyclin B by releasing ubiquitins from ubiquitin-cyclin B conjugates. Since p28 has many potential post-translational modification sites (data not shown here), it may also be abolished by modification soon after GVBD, resulting in abrupt degradation of cyclin B and thus the exit of the cell from M phase.

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