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In Vivo and *in Vitro* Effects of a Protease Inhibitor (E-64) on Cell Division of *Xenopus* eggs

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ABSTRACT—Microinjection of E-64 into single blastomeres of *Xenopus* embryos at the 2-cell stage arrested cell division. *In vitro* treatment of cultured individual cells at the blastular embryonic stage with E-64 c also arrested the cleavages. We examined the effects of E-64 on the proteasome and calcium-activated protease in *Xenopus* embryos *in vitro*. E-64 inhibited calpain-like protease activity. Calpain-like protease activity was stimulated during cleavage. These results indicated that a calpain-like protease might be involved in the cell division of early *Xenopus* embryos.

INTRODUCTION

After fertilization, the early embryonic development of *Xenopus* is characterized by a number of rapid synchronous cell cycles, and the embryogenetic events are precisely timed. The first cell division occurs about 90 min after fertilization, and the next 11 cell cycles each last approximately 25–30 min and comprise alternating S and M phases. After these 12 divisions, the cell cycles lengthen and become asynchronous (Newport and Kirschner, 1982a, b). There are three major control or transition points of the cell division cycles: the conversion of quiescent cells to a state of proliferation, the initiation of DNA synthesis by the growing cells, and the induction of mitosis of cells with newly replicated genomes (Dunphy and Newport, 1988; Stillman, 1996). Accumulated evidence indicates that regulation of the multiple processes involved in the G1 $\rightarrow$ S transition, i.e., mitosis, meiosis, gene transcription and the intercellular signal transduction pathway, requires the rapid elimination of key regulatory proteins or rate-limiting enzymes (Ciechanover, 1994; King, 1996; Hoyt, 1997; Sorimachi, 1997). It has been proposed that at least three different proteolytic pathways are involved in the degradative processes required to control the various steps in eukaryotic cell cycles: ubiquitin-proteasomes, calpains and lysosomal proteases. In previous studies, we have studied cathepsin L-like protease that are activated during the early embryogenetic stages of *Xenopus* (Miyata and Kihara, 1995; Miyata and Kubo, 1997). In the present study, we examined the effects of a thiol protease inhibitor, E-64, on cell division, in order to examine the roles of cysteine proteases in the cell cycle regulation of *Xenopus* eggs. We found that E-64 arrested cell division of *Xenopus* embryos at the 2-cell stage and cells isolated from the blastula embryo.

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MATERIALS AND METHODS

Embryos and microinjection

Eggs of *Xenopus* laevis were obtained by injection of a gonadotropic hormone and de-jellied with 2.5% sodium thioglycolate. The embryos were allowed to develop to the 2-cell stage at room temperature. trans-Epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E-64) was dissolved in dimethylsulfoxide (DMSO) and, in order to ensure that the effect of the DMSO was constant for each concentration of E-64 injected, the same volume of 100% DMSO was used for each injection. A 20 nl aliquot of the required E-64 solution or DMSO (control) was injected into one side of an embryo by inserting the tip of the micropipette into the region of the animal from above and pushing it until it reached the center of the blastomere.

The total volume of a *Xenopus* egg, which is 1.2–1.3 mm diameter, is about 1000 nl, and about 50% of this is composed of nonorganellar cytoplasm (Han *et al.*, 1992). At the 2-cell stage, the volume of the fluid cytoplasm of one blastomere is a quarter of the total volume of the egg (250 nl), and this volume was used to calculate the concentration of E-64 injected into each egg. Therefore, when the concentration of E-64 in the micropipette was 2.5, 5 or 15 ng/nl, the final cytoplasmic concentration was 200, 400 and 1,200 μg/ml, respectively.

Cell culture

Animal cap pieces were dissected from stage 8 blastulae. Single cells from the inner surface of the pieces were separated off by directing a gentle stream of calcium- and magnesium-free medium (50 mM phosphate buffer containing 35 mM NaCl and 1 mM KCl, pH 7.0) as described by Godsave and Slack (1989). Two or three cells were transferred into a well of a Terasaki plate filled with 10 μl of 2 mg/ml γ-globulin in a simple salt solution (NAM/2) and cultured for 20 h at 25°C.

Enzyme assays

Proteasome activity was determined using the method reported by Azuma *et al.* (1991) with modifications. The assay was carried out using a 200 μl 100 mM Tris-HCl buffer (pH8.2) containing 5 mM $MgCl_2$ with or without 2 mM ATP and 50 μM Suc-Leu-Leu-Val-Tyr-4-methylcoumaryl-7-amide (MCA) as the substrate. An aliquot (10 μl–20 μl) of an enzyme solution was added, and the reaction mixture was incubated at 37°C. After 1 hr, the proteolytic reaction was stopped

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by adding 100 µl 10% SDS and 2 ml 100 mM Tris-HCl buffer (pH 9.0), and the amount of 4-methylcoumaryl-7-amine released was measured fluorometrically (excitation at 360 nm, emission at 460 nm).

Calcium-activated protease activity was measured by using azocasein as the substrate and by means of a method described previously (Inomata *et al.*, 1984). Each 200 µl assay reaction mixture comprised 0.2% azocasein, 28 mM 2-mercaptoethanol and 100 µM Tris-HCl (pH 7.5) with or without $CaCl_2$ (0–100 mM). The reaction was initiated by the addition of 50 µ l of enzyme solution. After incubation at 30°C for 2 hr, the reaction was stopped by adding 200 µl 10% trichloroacetic acid. The mixture was then centrifuged at 3,000 rpm for 10 min, and the proteolytic activity was assayed by measuring the amount of digested products liberated into the trichloroacetic acid-soluble fraction using the reagent for protein assays from Bio-Rad.

Preparation of enzyme samples

In order to assay the proteasome activities of *Xenopus* embryos *in vitro*, aliquots of enzyme samples were prepared using the method reported by Tokumoto and Ishikawa (1993). Five-hundred embryos at the blastula stage were homogenized in 5ml 25mM Tris-HCl buffer (pH 7.9) containing 10 mM 2-mercaptoethanol and 20% glycerol. The homogenate was centrifuged at 36,000×g for 30 min at 4°C, and the clear supernatants were subjected to Sepharose 6B column (1×90 cm) gel filtration. Of the 700 µl-fractions collected, fraction No. 23 showed the highest proteasome activities. In order to assay the calpain activity of *Xenopus* embryos *in vitro*, a crude enzyme extract was prepared as described by Inomata *et al.* (1983). *Xenopus* embryos (500 embryos at the blastula stage) were homogenized in 5 ml 20 mM Tris-HCl (pH 7.5) containing 5 mM EDTA and 10 mM 2-mercaptoethanol, centrifuged at 17,000×g for 15 min, and then the calpain activity of the supernatant was assayed as described above.

RESULTS

In an attempt to determine whether cell division of early embryos was modified by proteases, we tested the effects of microinjecting the protease inhibitor E-64, which has been demonstrated to react exclusively with essential cysteine residues at the active sites of cysteine proteases *in vivo* and *in vitro* (Tamai *et al.*, 1986; Gour-Salin *et al.*, 1989). Inhibition of cell division by E-64 should confirm the type of cell cleavage-associated protease. Microinjection of 100ng/egg E-64 into one blastomere of a 2-cell- stage embryo inhibited cell cleavage. The uninjected sister blastomere developed normally until

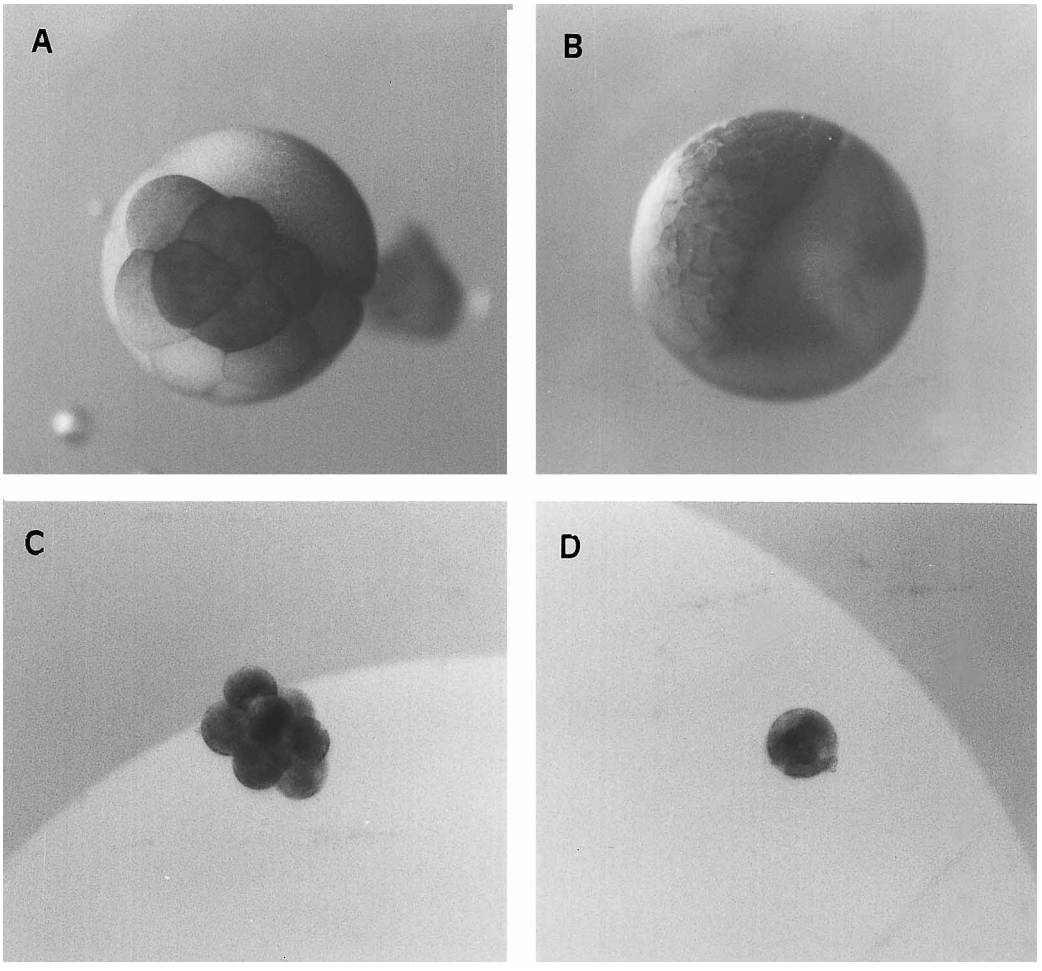


Fig. 1. Effects of microinjection of E-64 into developing *Xenopus* eggs and treatment of isolated cells with E-64. E-64 (100 ng/egg) was injected into one blastomere of a 2-cell stage embryo after the first cleavage. Figs. 1-A and 1B show embryos 2 and 4 h after the injection of E-64, respectively. Isolated cells of stage 8 blastulae were cultured in the medium for 20 hr. Fig. 1-C shows a blastomere that was cleaved in the medium. Fig. 1-D shows a blastomere in which cell division is inhibited by E-64 c in the medium.

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about the 32-cell stage within 2 hr of injection of E-64 into the other blastomere, whereas the injected blastomere was arrested at the 2-cell stage (Fig. 1-A). Fig. 1-B shows such embryos 4 hr after injection, and it is clear that the noninjected blastomere consisted of small normal-looking cells, whereas the injected blastomere was clearly arrested at the 2-cell stage.

We tested three different concentrations of E-64: 50, 100 and 300 ng/egg. Both DMSO alone and E-64 dissolved in DMSO caused similar abnormalities, which varied from complement arrest at the 2-cell stage to cells of irregular sizes due to abnormal cleavage and patchy pigmentation. About 20–25% of the embryos injected with various concentrations of E-64 and with DMSO alone stopped at the 2-cell stage and had died by the time the control sibling embryos developed to the early morula stage. Cell cleavage of about 30% of the blastomeres injected with E-64 and DMSO alone was slower than that of untreated sibling embryos, and some cells of the injected blastomeres became larger than normal. After the mid-blastula stage, the embryos with a noninjected blastomere consisting of small normal-looking cells and a injected blastomere arrested at the 2-cell stage were scored (Table 1). Injection of 50 ng/egg E-64 resulted in cleavage arrest in 33% of the injected embryos. Microinjection of E-64 into one blastomere caused complete arrest of the cell cycle in a concentration-dependent manner. Injection of 100 and 300 ng/egg resulted in cleavage arrest in 87% and 95%, respectively, of the treated embryos, whereas 36 of 54 (67%) of the embryos injected with 50 ng/egg exhibited normal development of the injected blastomeres. After injection of 100 and 300 ng/egg E-64, 13% and 5%, respectively, of the injected embryos developed normally. Control blastomeres were microinjected with an equivalent volume (20 nl/egg) of DMSO, which affected 13% of the injected embryos by arresting the cell cycle. However, 87% of the DMSO-injected embryos developed normally.

We carried out *in vitro* treatments of isolated cells with (2S, 3S)-trans-Epoxysuccinyl-L-leucylamido-3-methylbutane (E-64 c) in order to see more clearly the effects of E-64 c on cell division. Single cells from mid-blastula-stage embryos were able to divide in a non-nutritive medium (Fig. 1-C). Under the standard conditions of the present study, most cells divided between 4 and 10 times. Effects of the protease inhibitor on the division of these cells were studied (Table 2). A concentration of 400 μg/ml E-64 c resulted in inhibition of cleavage in 50% of individual cells, and a concentration of 800 μg/ml E64 c inhibited cleavage in 83% (Fig. 1-D).

To identify enzyme(s) that related to cell cleavage and were inhibited by E-64 in a concentration-dependent manner, the activities of two proteases in homogenates of embryos were measured *in vitro*. First, we measured proteasome activity (Table 3). To establish whether proteasome activity was present in the embryos, we prepared an embryo homogenate, subjected it to Sepharose 6B gel filtration and obtained a high molecular weight fraction containing proteasome activity. One gel filtration peak showed hydrolytic activity toward Suc-Leu-Leu-Val-Tyr-MCA in the presence of ATP. Adding ATP to the reaction mixture increased the proteasome activity to about twice that of the ATP-free reaction mixture. The ATP-activated proteasome activity was not inhibited by E-64, even at a concentration of 400 μg/ml (Table 3).

E-64 inhibits the activities of cysteine proteases, including cathepsin and calpain, and it has been reported that it

Table 1. Effects of microinjection of E-64 into developing *Xenopus* eggs.

E-64 and DMSO, at the indicated concentrations, were injected into the blastomeres of 2-cell stage *Xenopus* embryos and the effects were observed 3 hr later. The data presented are the numbers of embryos showing cell cycle arrest and normal development.

DMSO or E-64 (μg/ml)	Embryos arrested at 2-cell stage(%)	Embryos showing normal development (%)
DMSO	7 (13%)	45 (87%)
E-64		
200 (50 ng/egg)	18 (33%)	36 (67%)
400 (100 ng/egg)	47 (87%)	9 (13%)
1200 (300 ng/egg)	52 (95%)	3 (5%)

Table 2. Effects of E-64-c treatment on cells isolated from blastula embryos

Isolated cells from blastulae were cultured in a medium containing 2% DMSO and 100 μg/ml, 200 μg/ml, 400 μg/ml or 800 μg/ml E-64c. After 20 hr, cleavage of blastomeres were observed under a microscope.

DMSO or E-64c (μg/ml)	No. of non-cleavaged cells	No. of cleavaged cells
2% DMSO	16 (5.4%)	279 (95%)
100	24 (8.5%)	262 (92%)
200	70 (26%)	197 (74%)
400	117 (50%)	117 (50%)
800	217 (83%)	44 (17%)

Table 3. Effects of E-64 on proteasome and calcium-activated protease.

The enzyme activities were measured as described in MATERIALS AND METHODS. The activities of the control reaction mixtures containing no E-64, ATP or $CaCl_2$ were considered to be 100%, and the activities in the presence of ATP or $CaCl_2$ and E-64 and ATP or $CaCl_2$ were expressed relative to these values.

Class of protease	E-64	Addition	Activity (%)
Proteasome	–		100
	–	ATP	210
	400 μg/ml	ATP	202
Calpain-like protease	–		100
	–	5μM Ca^{2+}	380
	–	5μM Ca^{2+}	820
	200 μg/ml	5μM Ca^{2+}	130
	400 μg/ml	5μM Ca^{2+}	98
	200 μg/ml	5μM Ca^{2+}	162
	400 μg/ml	5μM Ca^{2+}	93

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preferentially inhibits the activity of calcium-activated proteases, although this effect was not specific (Inomata *et al.*, 1984; Gour-Salin *et al.*, 1994). Therefore, we examined the effect of E-64 on the activity of a calcium-activated protease in a *Xenopus* embryo homogenate (Table 3). Adding 5 μM $CaCl_2$ to the reaction mixture resulted in a 3.8-fold increase in activity compared with that of the mixture without $CaCl_2$, and the activities of the calcium-activated protease in both mixtures were inhibited by 400 μg/ml of E-64. Addition of 5mM $CaCl_2$ led to 8-fold higher activity compared to that without $CaCl_2$, and the activities of the calcium-activated protease were also inhibited by 400 μg/ml E-64. In light of its requirement for calcium and its sensitivity to E-64, it is likely that this proteolytic activity was mediated by a member of the calpain family of proteases.

The changes in calpain-like protease activities were determined during the early stage of embryo development (Table 4). A gradual increase in cystein protease activity occurred after fertilization. Micro calpain-like protease activity was enhanced by about 200–250% in blastular or gastrula embryo as compared to that in unfertilized eggs. Milli calpain-like protease activity was increased by about 500% in blastular or gastrula embryos in comparison with that in unfertilized eggs.

Table 4. Changes in proteolytic activity during development
Embryos were collected at different stages of development. *Xenopus* eggs and embryos (100 eggs and 100 embryos) were homogenized in 1 ml 20 mM Tris-HCl (pH 7.5) containing 5 mM EDTA and 10 mM 2-mercaptoethanol, centrifuged at 17,000 × g for 15 min, and then the cystein protease and activity of calcium-activated protease in the supernatant was assayed as described in the MATERIALS AND METHODS section. The activity of the unfertilized eggs containing no $CaCl_2$ was considered to be 100%, and the activities of unfertilized eggs, blastula embryos and gastrula embryos in the presence of $CaCl_2$ were expressed relative to that value.

Calcium concentrations	Activity (%) in unfertilized eggs	Activity (%) in blastula embryos	Activity (%) in Gastrula embryos
0	100	130	147
5 μM	79	210	267
5 mM	85	495	492

DISCUSSION

In an attempt to identify the protease(s) involved in the cell cycle in early *Xenopus* embryo development, we studied the effects of E-64 on cell cleavage in development. Equivalent concentrations of E-64 inhibited the cell cleavage of early embryos both *in vivo* and *in vitro*, and they inhibited calcium-dependent neutral protease activity in homogenates of the embryos *in vitro*. Calpain-like protease activity increased after fertilization.

There is evidence suggesting that calpains and cysteine proteases degrade cell cycle-related proteins, and benzyloxycarbonyl-Leu-Leu-Tyr diazomethyl ketone (ZLLY-DMK), which is a relatively selective calpain inhibitor, has been found to inhibit the growth of human TE2 and C-33 cells (Mellgren, 1994). Moreover, exposure to ZLLY-DMK during the late G1 phase inhibits progression of the S phase of serum-stimulated WI-38 fibroblasts (Zhang, *et al.*, 1997; Mellgren, 1997). Since cleavage of p53 by a calpain was inhibited by ZLLY-DMK, the fibroblasts could not proceed from the G1 phase to the S phase (Zhang, *et al.*, 1997), suggesting that the activity of a calpain-like ptotease is responsible for the degradation of p53 observed during the late G1 phase.

With respect to the promotion of the M phase, E-64 has been reported to arrest the proliferation of human epidermoid carcinoma A431 cells during metaphase (Shoji-Kasai *et al.*, 1988). In addition, E-64 has prevented spindle formation during mouse meiotic maturation (Hashimoto, *et al.*, 1990), and microinjection of calpain into PtK1 cells during interphase has promoted the onset of the metaphase (Schollmeyer, 1988). Cyclins A and B promote the S and M phases, and degradation of these cyclins is required for cells to exit the M phase (Minshull *et al.*, 1989; Sherr, 1993 and 1996). When calcium was added to the cytosol of *Xenopus* embryos for an *in vitro* reaction of the degradation of cyclin B, degradation of cyclin B occurred in the reaction mixture (Funakoshi *et al.*, 1999). These findings indicate that cysteine proteases, including calpains, are important for the transition from the G1 phase to the S phase and for the progression of the M phases of the cell cycle (Zhang, *et al.*, 1997; GMellgren, 1997; Shoji-Kasai *et al.*, 1988; Hashimoto, *et al.*, 1990).

E-64, however, has also been reported to have no effect on intracellular calcium-activated neutral protease in rabbit erythrocytes (Hayashi *et al.*, 1991), and relatively high concentrations (50–400 μg/ml) of E-64 are needed in these experimental systems to exert the inhibitory effects on the cell cycle described above (Shoji-Kasai *et al.*, 1988; Hashimoto *et al.*, 1990; Mellgren *et al.*, 1997). Although we also observed that the inhibitory effect of E-64 *in vivo* and *in vitro* occurred at relatively high concentrations (400–800 μg/ml), a calpain-like protease may be responsible for processing the cell cycle in the early *Xenopus* embryo.

As the early embryonic cell cycle in *Xenopus* consists of only S and M phases and does not include the G 1 or G 2 phases (Newport and Kirschner, 1982a, b), arrest of the cell cycle of the *Xenopus* embryos by E-64 is unrelated to the inhibition of proteolysis at the G 1 → S phase transition. The arrest of the cell cycle of the *Xenopus* embryo by E-64 may be related to the preservation or progression of the M phase, as reported previously in studies that used cultured cells (Shoji-Kasai *et al.*, 1988; Hashimoto, *et al.*, 1990).

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