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Occurrence and the state of a tudor repeat protein in the development of *Xenopus laevis*

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Abstract

The *Xenopus* tudor repeat (Xtr) protein of the unfertilized and fertilized eggs at different intervals, and embryos of different stages was compared by immunoprecipitating with anti-Xtr monoclonal antibody, followed by Western blotting using anti-Xtr antibody. Besides the Xtr of 270 kDa, another band of Xtr below 150 kDa, was detected reproducibly after fertilization of the eggs. This result suggested that a part of Xtr was degraded after fertilization. The screening profile of the existence of Xtr and the distribution of degradation from unfertilized eggs to the nerula stage embryos showed that in the unfertilized eggs there was no occurrence of degradation. But it was visualized just after 20-minutes of fertilization and lasted up to the gastrula stage. Interestingly, the degradation could not be detected beyond this stage of development. Probably, the Xtr protein was degraded following the activation of the eggs because previous investigations suggested that Xtr acted after fertilization or activation of the eggs.

INTRODUCTION

The African clawed frog, *Xenopus laevis,* is an excellent model animal for developmental and molecular biology experiments. Marteil *et al.* (2009) reported that the competence of oocytes depends on numerous processes taking place during the whole oogenesis, but its final steps such as oocyte maturation, seem to be of key importance. Modern approaches such as proteomic analysis demonstrate novel proteins, which are involved in oocyte development (Marteil *et al.*, 2009). However, no data is available regarding the status and relation of the germline cell and early embryonic cell-specific proteins to the developmental stages in *X. laevis*.

To find a clue to the understanding of the biological question how germline cells differentiate and develop separately from somatic lineage, Ikema *et al.* (2002) identified a novel gene, *Xtr* (*Xenopus* tudor repeat) in *X. laevis*. The transcriptional and translational products of *Xtr* exclusively occur in germline cells as well as early embryonic cells as maternal factors, but not in the adult somatic cells (Ikema *et al.* 2002; Hiyoshi *et al.*, 2005). Xtr protein contains plural tudor domains, which are also found in *Drosophila* Tudor (Golumbeski *et al.*, 1991) and is involved in both abdominal segmentation of the embryo and germ cell specification (Boswell & Mahowald, 1985). In mice, a representative to mammals, the tudor domain-containing proteins such as Tdrd1, Tdrd5, Tdrd6, Tdrd7, and Tdrd9 occur in germinal granules of spermatogenic cells and are involved in the maintenance of germinal granule architecture and spermatogenesis (Chuma *et al*., 2003, 2006; Hosokawa *et al*., 2007; Shoji *et al*., 2009; Vasileva *et al*., 2009; Tanaka *et al*., 2011; Yabuta *et al*., 2011). It has been reported that most of these tudor proteins associate with mouse Piwi proteins, Mili, Miwi, and/or Miwi2 in an sDMA-dependent manner (Shoji *et al*., 2009; Vagin *et al*., 2009; Vasileva *et al*., 2009; Kirino *et al*., 2010).

Recently, some investigators (Shoji *et al*., 2009; Tanaka *et al*., 2011; Yabuta *et al*., 2011) have shown that the loss-of-function of Tdrd1, −5, −7, and −9 by targeting their genes causes the upregulation of transposable elements, and probably these proteins play an important role in retrotransposon silencing. Conversely, the disruption of the *Tdrd6* gene does not cause significant upregulation of retrotransposon activity (Vasileva *et al*., 2009) and the exact function of Tdrd6 is still unknown. However, to investigate the role of the Xtr (Hiyoshi *et al*., 2005), they produced an anti-Xtr rabbit polyclonal antibody and microinjected it into *Xenopus* eggs and embryos for specifically inhibiting the function of Xtr and demonstrated that the loss-of-function of Xtr in the fertilized eggs resulted in the arrest of the cleavage. These results suggest that the karyokinesis of at least early embryonic cells is regulated by a unique mechanism in which Xtr is involved. Later, Mostafa *et al*. (2009) proved that Xtr coexists with another germ cell protein, FRGY2 by constituting an mRNP particle and the translational products of Xtr-associated mRNAs play crucial roles in karyokinesis progression and in germ cell development. Subsequently, Ohgami *et al*. (2012) demonstrated that that Xtr regulates the translation of XL-INCENP mRNA through its 3'UTR during meiotic progression of oocyte. The present study focuses on the occurrence and state of the protein in the developmental stages ranging from unfertilized eggs to some advanced stages of *Xenopus.*

MATERIALS AND METHODS

Preparation of eggs and embryos: Sexually matured South African clawed frogs, *Xenopus laevis*, were purchased from dealers in Hyogo Prefecture, Japan. Matured eggs and fertilized eggs or embryos were prepared as described by Ikema *et al*. (2002). Jelly envelopes were removed from the matured eggs and fertilized eggs by treatment with 2% of cysteine (alkalinized by NaOH; pH 8.0) for 5–10 min and the dejellied eggs were incubated in 1× Marc's Modified Ringers (MMR; 100 mmol/L NaCl, 2 mmol/L KCl, 1 mmol/L $MgSO_4 \cdot 7H_2O$, 2 mmol/L $CaCl_2$, 5 mmol/L Tris-HCl; pH 7.4) until use.

Antibodies: Detection of Xtr was carried out by using anti-Xtr antisera, which were produced against bacterially expressed Xtr (Hiyoshi *et al.*, 2005). To obtain monoclonal antibody (mAb) against the Xtr, female BALB/c mice (6–7 weeks old) were immunized with a bacterially expressed Xtr (Hiyoshi *et al.*, 2005) according to the protocol described by Kubo *et al*. (1999). Hiyoshi *et al*. (2005) isolated popliteal lymph node cells three days after the booster immunization, and fused with PAI myeloma cells. They identified hybridomas producing anti-Xtr antibodies by the enzyme-linked immunosorbent assay using microtiter plates precoated with a bacterially expressed Xtr. In this process, aliquots of the culture supernatant from the established clones were used to determine the isotype (IgG2a) of the antibodies with a Mouse Monoclonal Antibody Isotyping Kit (GE Healthcare, Sweden). The hybridoma cells were transplanted into the body cavity of pristane-primed nude mice, and the ascites fluid was collected. Monoclonal antibodies were purified from the ascites by using rProtein A Sepharose Fast Flow (GE Healthcare). Mouse IgG2a, κ (UPC-10; Sigma, USA) was used as a normal mouse IgG (NMI) and for the examination of Xtr-interacting protein, the purified monoclonal antibody was covalently conjugated with Protein ASepharose. Briefly, after binding 2 mg antibody to 1 mL of rProtein A Sepharose Fast Flow, the antibody-Protein A complex was cross-

linked by treatment with dimethyl pimelimidate (Pierce, USA), according to the protocol of Harlow and Lane (1988). On the other hand, for cloning of Xtr-interacting mRNAs, the mAb (10 μg) was incubated with rProtein A Sepharose Fast Flow (50 μL) for 1 h and the gel was used after washing it.

Immunoprecipitation and Immunodetection of Xtr Protein: Twenty dejellied unfertilized eggs and embryos of different stages were separately homogenized in 1.5 ml homozenizing buffer (1mM ethylenediaminetetraaceticacid (EDTA), 150 mMNaCl, 0.1% NP-40, 1mM phenylmethanesulfonylfluoride (PMSF) 1μg/mL N-tosyl-Lphenylalanylchloromethyl ketone (TPCK), 1μg/mL Ntosyl-L-lysylchloromethyl ketone (TLCK), 10 mMTris-HCl; pH 7.5) and centrifuged at 16000 *g* for 10 min and collected 1 ml of the supernatant after centrifugation and used it for immunoprecipitation. Xtr was immunoprecipitated with ant-Xtr monoclonal antibody cross-linked on Protein A Sepharose beads. However, to check the state of the protein, Xtr among the unfertilized and fertilized eggs at different intervals by immunoprecipitating Xtr with anti-Xtr monoclonal antibody the anti-Xtr rabbit antibody which is bound to Protein A Sepharose beads because antibodies (around 50kDa) are detected by Western blotting. The binding proteins were eluted from the gels by boiling those in Laemmli's sample buffer (Laemmli, 1970) and the eluate was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDSPAGE) for Western blot analyses.

SDS-PAGE and Western blotting: To detect the Xtr, the samples were electrophoresed in a 5% gel sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and blotted onto sheets (Immobilon P; Millipore, USA) according to the manufacturer's methods. The transblotted sheets were incubated with 1:1000 diluted anti-Xtr rabbit antiserum overnight, followed by treatment of them with 1:1000 diluted alkaline phosphatase-conjugated goat anti-rabbit IgG (Chemicon International, USA). Detection of the signals was carried out by using 4-nitroblue tetrazoliumchloride/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) (Roche, USA), according to the methods of the supplier.

RESULTS AND DISCUSSION

In the investigation of the occurrence of the Xtr in the unfertilized eggs, fertilized eggs and in some stages during embryogenesis observed by immunoprecipitation and Western blot analyses using anti-Xtr antibody. Western blot analysis probed with anti-Xtr rabbit antiserum clearly showed the presence of Xtr in the anti-XtrmAb-immunoprecipitated sample but not in the NMI immunoprecipitated sample (Fig.1). This result suggested that Xtr coimmunoprecipitated with anti-Xtr monoclonal antibody and was eluted specifically from antibody-conjugated Protein-A Sepharose beads without contamination of non-specifically binding proteins on the Sepharose beads.

When occurrence of the Xtr during oogenesis and embryogenesis was observed by Hiyoshi *et al*. (2005) by immunoprecipitation and Western blot analyses using anti-Xtr antibody, a considerable amount of Xtr was found in unfertilized eggs and this amount was kept constant until the tailbud stage and decreased thereafter.

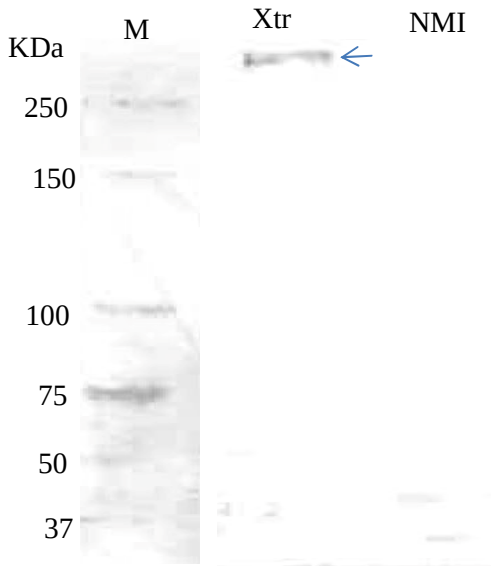

Fig. 1. Existence of intact Xtr in the unfertilized eggs. M=Protein Marker, the arrowhead in the left lane shows the presence of Xtr of 270 KD in the eggs immunoprecipitated with anti-Xtr antibody, while in the Normal Mouse IgG (NMI)-immunoprecipitated sample which served as a control

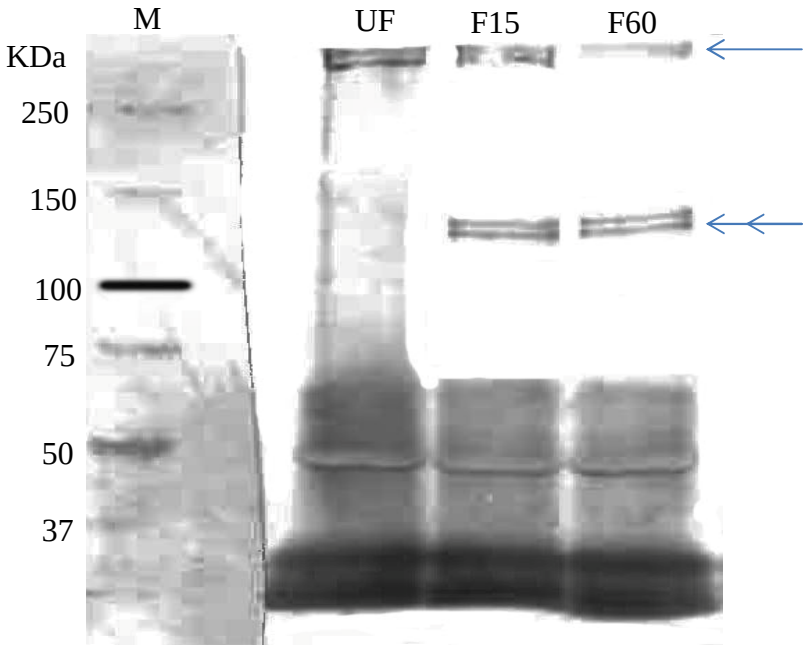

Fig. 2. Comparison of the existence of Xtr between unfertilized and fertilized eggs. M, Protein Marker; UF, Unfertilized eggs; F15, Eggs 15mins after fertilization; F60, Embryos 60 mins after fertilization. The upper arrowhead indicates the intact Xtr, while the lower arrowhead shows the degraded form of Xtr

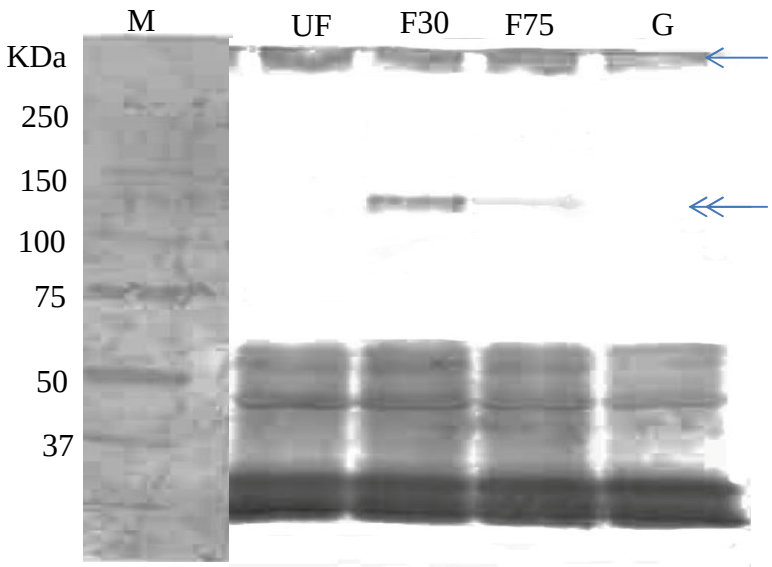

Fig. 3. Comparison of Xtr degradation among fertilized eggs. M, Protein Marker; UF, Unfertilized eggs; F30, Embryos 30mins after fertilization, F75 and G, Gastrula. The upper arrowhead indicates the intact Xtr, while the lower double arrowhead shows the degraded form of Xtr

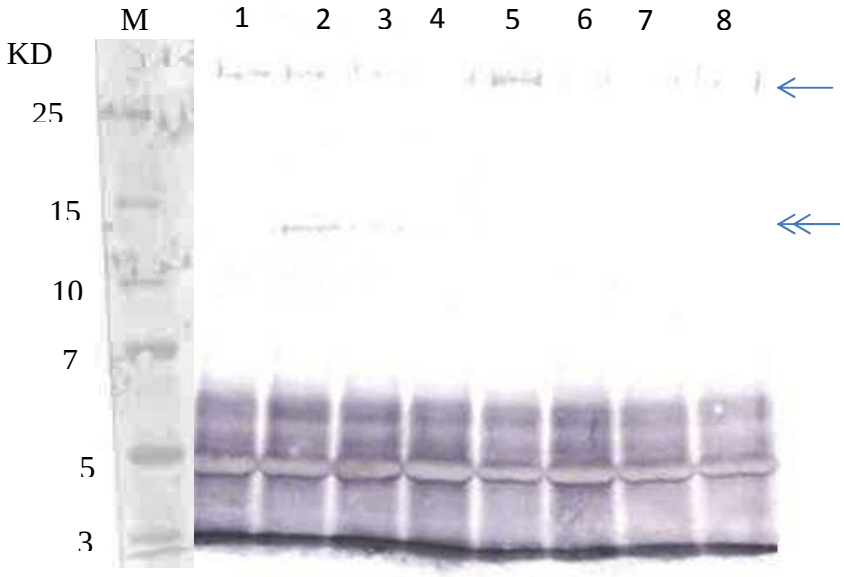

Fig. 4. Profile of the state of Xtr and its degradation at various stages of *Xenopus* eggs or embryos. M=Protein Marker; 1, Unfertilized eggs; 2, Eggs twenty mins after fertilization; 3, Embryos of two-cell stage; 4, Middle of two cell stage; 5, Morula stage; 6, Mid Blastula, 7; Gastrula and 8, Nerula stage embryos. The upper arrowhead indicates the intact Xtr, while the lower double arrowhead shows the degraded form of Xtr

To study the state of the protein, Xtr was compared among the unfertilized and fertilized eggs at different intervals by immunoprecipitating Xtr with anti-Xtr monoclonal antibody, followed by Western blotting using anti-Xtr antisera, another band of Xtr below 150 kDa, which appeared after fertilization (Fig. 2, double arrowhead), was detected reproducibly. This result suggested that a part of Xtr degraded after fertilization. However, this result was a preliminary observation. Further investigations including the characterization of this extra band would facilitate the understanding of the biological meaning of the degradation of Xtr protein.

To further investigate the state of Xtr at different developmental stages beyond fertilization of the eggs, the phenomenon of degradation of Xtr was observed. Interestingly, this degraded form of Xtr was detected only in the fertilized eggs just after fertilization, which occurred up to several developmental stages of the embryos. The occurrence of Xtr degradation in the fertilized eggs was recorded in varied degrees while no degradation was observed in the unfertilized eggs. The degree of degradation was higher after 30 minutes of fertilization and gradually lower upto the gastrula stage (Fig. 3). The profile of the existence of Xtr and the distribution/stretch of degradation from unfertilized eggs to the nerula stage embryos demonstrated that in the unfertilized eggs there was no occurrence of degradation but the degradation was visualized just after 20-minutes of fertilization and lasted up to the gastrula stage but it could not detected in the nerula stage (Fig. 4).

However, this degraded Xtr was absent in unfertilized eggs. This degraded form of Xtr in fertilized eggs seemed to be the active form of Xtr, which interacts with FRGY2 and thus it is important for understanding the exact function of Xtr protein. Although the active state of Xtr state is yet to be known, it is possible that Xtr protein is degraded following the activation of the eggs because previous investigations suggested that Xtr acts after fertilization or activation of the eggs. Therefore, the degraded Xtr might be the active form of Xtr protein, which upon fertilization interacts with FRGY2 to activate the translation of the FRGY2-repressed maternal mRNAs. Following this activation of the translation of the maternal mRNAs both chromosome condensation and microtubule assembly might occur for the proper karyokinesis during cell division, and thus the translational products of these mRNAs contribute to the cell cycle progression as well as in germ cell development (Mostafa, 2009).

Although this observation of the degradation of Xtr protein is preliminary, it might provide a clue to understanding the molecular mechanisms of the function of Xtr protein in future. Therefore, further analyses on the structure of this degraded Xtr protein and what causes this degradation needs to be investigated.

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