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THE CYTOSOLIC CHAPERONIN SUBUNIT TRIC-P5 BEGINS TO BE EXPRESSED AT THE TWO-CELL STAGE IN MOUSE EMBRYOS

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The cytosolic chaperonin TRiC is a large protein complex involved in the folding of newly synthesized actin and tubulin. The fertilization of the mouse oocyte is followed by a remodelling of the actin and tubulin filaments. The TRiC subunit TCP1 is expressed only from the 4-cell stage on, even though actin and tubulin are synthesized in the previous stages. We investigated the onset of synthesis of another subunit, TRiC-P5, during early mouse embryogenesis. We report that TRiC-P5 is synthesized at the 2-cell stage in an alpha-amanitin sensitive manner. Thus, it is expressed before TCP1 and is one of the first proteins to be synthesized after zygotic genome activation.

Molecular chaperones constitute a superfamily of proteins containing heat-shock and non heat-shock proteins, that are able to fold or to assemble nascent polypeptides. Members of the hsp60 family form large protein complexes. These chaperonins are double-ring complexes containing six to nine subunits per ring (1-4). Usually, the chaperonin rings are formed by a single protein (1, 5-7). Up to now, only one chaperonin has been described in the cytosol of eukaryotic organisms. This eukaryotic cytosolic chaperonin has the same organization as its prokaryotic counterparts, but the complex is an hetero-oligomer composed of related proteins of molecular weight ranging from 52 to 65 kDa (3,8,9). This complex has been called TRiC for TCP1-Ring Complex (3) or CCT for Chaperonin-Containing TCP1 (9), since many reports have identified the murine t-complex polypeptide 1 (TCP1) as one of its subunits (2,10,11). The *Tcp1* gene is located on mouse chromosome 17 within the t-complex (12). This locus codes for many genes implicated in early embryogenesis and in the phenomenon of male-specific transmission ratio distortion (13).

TRiC has been shown to facilitate the folding of newly-synthesized actin and tubulin *in vitro* and *in vivo* (3,10,14,15). In yeast, the TRiC subunits are essential for cell viability. The phenotype of yeast in which these proteins are mutated indicates that they are critical for tubulin and actin function *in vivo* (16-20). One of the TRiC

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subunits, TRiC-P5, is a 63-kDa protein expressed mainly in testis of adult animals (21). On two-dimensional gels, the protein migrates as isovariants between pI 6.6-7.0. It is strongly conserved throughout evolution. For example, there is an overall 59% amino acid identity between mouse and ciliates (22). Unlike the *Tcp1* gene, the *TRiC5* gene is not localized within the t-complex in the mouse genome. It maps to chromosome 3 which is not known to be implicated in defects in embryogenesis (23,33). We have found that cell lines originating from undifferentiated leukemic cells contain high levels of TRiC-P5 mRNA, and that this mRNA is down-regulated when differentiation is induced *in vitro* by various chemicals (Joly *et al.* unpublished results). These observations suggest an involvement of TRiC-P5 in cell differentiation or development

Considering that actin and tubulin networks are extensively reorganized in oocytes after fertilization (24,25), that they continue to be synthesized, and that TRiC folds these proteins, we were interested in looking at TRiC-P5 expression in preimplantation mouse embryos. In the present paper, we show that synthesis of TRiC-P5 begins at the 2-cell stage, in an alpha-amanitin sensitive manner.

MATERIALS AND METHODS

Embryo collection and labeling. Mouse embryos at 1-, 2-, 4-, 8-cell, and blastocyst stages were obtained from C57BL6 x C3H F1 superovulated female mice (26). For labeling, mouse embryos were washed in medium 2 (M2) and incubated for one hour in 100 µL of medium 16 (M16) containing 1 mCi/mL of [^{35}S]Methionine (Amersham) at 37°C.
For alpha-amanitin treatment, fertilized eggs at the 1-cell stage were recovered from the oviducts of female mice 10-12 h after the ovulating injection of hCG (human chorionic gonadotrophin), washed with M2 medium and incubated *in vitro* for various times in M16 medium with or without 11 µg/mL alpha-amanitin. Similarly, 29 h after recovery and culture in M16 medium, embryos at the late-2-cell stage were incubated *in vitro* for 19 h in M16 medium containing 11 µg/mL alpha-amanitin. Newly-synthesized proteins were labeled 2 h with [^{35}S]Methionine at 37°C.

Cell culture. Burkitt lymphoma Raji cells were propagated in suspension and cultured in RPMI-1640 (BRL) supplemented with 10% fetal calf serum (Flow Laboratories). For labeling, the cells were incubated overnight at 37°C with 10 µCi/mL of [^{35}S]Methionine.

Immunoprecipitation. Twenty to fifty embryos were collected from each stage and labeled as described above. After washing three times in M2 medium, the embryos were lysed in 800 µL of lysis buffer (20 mM KPO_4 (pH 7.5), 0.1 M NaCl, 0.5% Na dexoycholate, 1% Triton x-100, 0.1% SDS, 20 mM methionine, 1 mM PMSF, 1 mM benzamidine, 25 µg/mL leupeptine, 25 µg/mL aprotinine). For Raji cells, 5.3 x 10^6 labeled cells were washed in PBS buffer and lysed. Samples were sonicated 3 times at 6 microns for 30 sec and kept under agitation for 1 h at 4°C. After ultracentrifugation at 40,000 g for 1 h, 700 µL of supernatant from embryo lysate, or 25 µL of Raji lysate was incubated with 10 µL of preimmune serum during 16 h at 4°C. All the incubation steps were done under agitation in a Lab-Quake apparatus. After addition of 150 µL of 20% IgGSorb (The Enzyme Center Inc.), the incubation was continued for 1 h at room temperature. IgGSorb was removed by a 20 min centrifugation at 16,000 g. Each supernatant was incubated overnight at 4°C with the specific anti-TRiC-P5 antibody (27), followed by addition of 50 µL of the IgG-Sorb suspension, and incubated again for 1 h at 22°C. The IgG-Sorb was then washed 3 times with lysis buffer, and 3 times with PBS containing 1 mM PMSF. The bound proteins were eluted by boiling the IgG-Sorb in 75 µL of gel sample buffer (28).

Protein analysis. Immunoprecipitated proteins were separated on a 8% SDS-PAGE (28). After electrophoresis, gels were treated with En3Hance and dried. Labeled proteins were visualized by fluorography.

RESULTS AND DISCUSSION

TRiC-P5 synthesis during embryogenesis

We have investigated at which stage TRiC-P5 starts to be synthesized during mouse embryo development. Preimplantation mouse embryos at various stages were collected from superovulated mice and labeled *in vitro* with [^{35}S]methionine. Stages were determined according to the time post-hCG injection and by visual examination under a dissecting microscope. After a short labeling period of 1 h, the newly-synthesized labeled proteins were immunoprecipitated with an antiserum against TRiC-P5 that was raised against the C-terminus of the protein. This antiserum detects a 63-kDa single band in Western blot analysis and immunoprecipitates newly-synthesized TRiC-P5 (27). As a positive control, Raji cells were labeled and submitted to the same immunoprecipitation procedure.

A single protein with the same MW as TRiC-P5 was specifically immunoprecipitated from Raji cells with the TRiC-P5 antiserum (Fig. 1, compare lanes 1 and 2), indicating that this serum is specific for TRiC-P5. No newly-synthesized proteins were immunoprecipitated from the 1-cell stage embryos (Fig. 1, lane 3). A polypeptide with the same MW as TRiC-P5 was visible in the 2-, 4-, 8-cell and

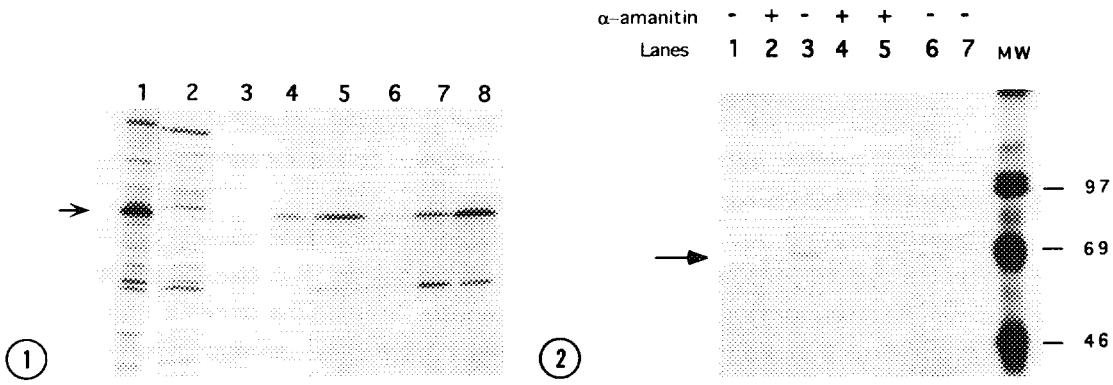

Fig. 1. SDS-PAGE of newly synthesized TRiC-P5 in preimplantation mouse embryos. Fertilized eggs were labeled *in vitro* for one hour and immunoprecipitated with an antiserum against TRiC-P5 (lanes 3, 4, 5, 6, 7, 8). Lane 3, 1-cell stage (50 embryos); lanes 4 and 5, 2-cell stage (50 embryos); lane 6, 4-cell stage (54 embryos); lane 7, 8-cell stage (21 embryos); lane 8; blastocyst stage (36 embryos). Raji cells were immunoprecipitated with the antiserum against TRiC-P5 (lane 1) or with a preimmune serum (lane 2) as a positive control. TRiC-P5 position is indicated by an arrow.

Fig. 2. Synthesis of TRiC-P5 in alpha-amanitin-treated preimplantation mouse embryos. Fertilized eggs at the 1-cell stage (lanes 1, 2, 3, 4, 6, 7) were incubated *in vitro* in presence (+) or in absence (-) of alpha-amanitin. Fifty embryos were immediately labeled (lane 1) or cultured for a further 26 h (lanes 2, 3, 7); or 48 h (lanes 4, 6) before a 2-h labeling period. Fifty embryos cultured *in vitro* to the late 2-cell stage were incubated for a further 19 h in presence of alpha-amanitin (+) before labeling (lane 5). Newly synthesized TRiC-P5 was immunoprecipitated with a specific antiserum and analyzed by SDS-PAGE. Labeled molecular weight standards (MW). TRiC-P5 position is indicated by an arrow.

blastocyst stage samples (Fig. 1, lanes 4, 5, 6, 7, 8). A polypeptide band migrating at 45 kDa was non-specific as it was immunoprecipitated as well by the preimmune serum (data not shown). These results indicate that the synthesis of TRiC-P5 starts at the 2-cell stage and is maintained at least up to the blastocyst stage.

Effect of alpha-amanitin on TRiC-P5 synthesis.

The first cell cycle in the mouse embryo is under maternal control, and the embryonic genome is activated at the 2-cell stage. At this stage, transcriptional activation is involved in replacing maternal transcripts that are common to both oocyte and early embryo, as well as generating novel ones that are likely to be involved in early embryogenesis. Alpha-amanitin inhibits the synthesis of a number of proteins that are thought to be products of zygotic gene activation (29,30). It prevents the 2- to 4-cell stage transition. To determine if the synthesis of TRiC-P5 at the 2-cell stage was under maternal or zygotic control, 1-cell stage mouse embryos were collected and incubated *in vitro* for various times in presence of alpha-amanitin, before labeling with [^{35}S]methionine.

Results with embryos cultured *in vitro* without alpha-amanitin were similar to those described with *in vivo* embryos. No proteins were immunoprecipitated from the 1-cell stage embryos (Fig. 2, lane 1). A single polypeptide migrating at 63 kDa was immunoprecipitated from embryos that had reached the 2- and 4-cell stages *in vitro*, in absence of alpha-amanitin (Fig. 2, lanes 3 and 6). The 63-kDa band was absent from embryos cultured during this period in presence of alpha-amanitin (Fig. 2, lanes 2 and 4). Addition of alpha-amanitin prior to mid-2-cell stage completely blocked the division of the embryos to the 4-cell stage (results not shown). Addition of alpha-amanitin after 39 h post-hCG injection allowed division of the embryos to the 4-cell stage, however the synthesis of TRiC-P5 was inhibited (Fig. 2, lane 5).

These results indicate that TRiC-P5 synthesis at the 2-cell stage is under the control of the zygotic genome as it is alpha-amanitin sensitive. It is thus the product of one of the first novel transcripts during embryogenesis. Among the proteins starting to be expressed at this stage of development few have been characterized. The molecular chaperone hsp70 is one of them (30). There is also synthesis of the 70-kDa transcription-requiring complex (TRC) (31). TRC is composed of a set of three alpha-amanitin sensitive proteins of 73, 70 and 68 kDa. These three proteins are present both in the cytoplasm and in the nucleus where a portion of these proteins is associated with a 2% Triton X-100 insoluble fraction (31). TRiC-P5 shares the same cellular localization, is in the same range of molecular weight and pI, and like TRC part of TRiC-P5 remains insoluble in presence of Triton X-100 (27). However TRiC-P5 is probably not one of the TRC, since these proteins are transiently synthesized at the 2-cell stage (31), while TRiC-P5 synthesis is maintained.

The fertilization of the mouse oocyte is followed by a major reorganization of the cytoskeleton (25). Actin and tubulin are expressed at the 1-cell stage from maternal RNA, and by the 2-cell stage their synthesis is under zygotic control (32). Our results

demonstrate that TRiC-P5 is not synthesized before the 2-cell stage. It is possible that newly-synthesized actin and tubulin are folded at the 1-cell stage via an other mechanism, or that TRiC-P5 is present in the unfertilized oocyte and do not need to be synthesized before the 2- and 4-cell stages.

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