Differential Accumulation of Ribonucleotide Reductase Subunits in Clam Oocytes: The Large Subunit Is Stored as a Polypeptide, The Small Subunit as Untranslated mRNA

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Abstract. Within minutes of fertilization of clam oocytes, translation of a set of maternal mRNAs is activated. One of the most abundant of these stored mRNAs encodes the small subunit of ribonucleotide reductase (Standart, N. M., S. J. Bray, E. L. George, T. Hunt, and J. V. Ruderman, 1985, J. Cell Biol., 100:1968-1976). Unfertilized oocytes do not contain any ribonucleotide reductase activity; such activity begins to appear shortly after fertilization. In virtually all organisms, this enzyme is composed of two dissimilar subunits with molecular masses of ~44 and 88 kD, both of which are required for activity. This paper reports the identification of the large subunit of clam ribonucleotide reductase isolated by dATP-Sepharose chromatography as a relatively abundant 86-kD polypeptide which is already present in oocytes, and whose level remains constant during early development. The enzyme activity of this large subunit was established in reconstitution assays using the small subunit isolated from embryos by virtue of its binding to the anti-tubulin antibody YL 1/2.

Thus the two components of clam ribonucleotide reductase are differentially stored in the oocyte: the small subunit in the form of untranslated mRNA and the large subunit as protein. When fertilization triggers the activation of translation of the maternal mRNA, the newly synthesized small subunit combines with the preformed large subunit to generate active ribonucleotide reductase.

The eggs and mature oocytes of marine invertebrates contain a set of messenger RNAs which are not translated to a significant extent until after fertilization (for review see references 4 and 18). Translation of these maternal mRNAs is essential for early development, as shown by the inhibition of cell division by protein synthesis inhibitors (II, 25, 30). One of the most abundant of these messages in both clams and sea urchins encodes a polypeptide of 42 kD which we previously identified as the small subunit of ribonucleotide reductase (23). Ribonucleotide reductase converts ribonucleoside diphosphates into deoxyribonucleoside diphosphates and thus provides the precursors for DNA synthesis (for review see reference 24). Both Escherichia coli and mammalian ribonucleotide reductases are tetrameric enzymes containing two types of subunits of the structure α2β2. The large subunit (M1 or B1, 84-88 kD) contains nucleotide-binding sites that regulate enzyme activity, whereas the small subunit (M2 or B2, 42-44 kD) contains an iron center and the tyrosyl-free radical required for ribonucleotide reduction. Both subunits are required to form the catalytic site, and no enzymatic activity is shown by either isolated subunit (24).

Earlier studies of ribonucleotide reductase in sea urchins showed that activity of this enzyme increased greatly after fertilization, and that the increase depended on protein synthesis, but it was not known whether this was due to synthesis of an activator or of the enzyme itself (17). Although clam M2 synthesis accounts for ~5% of the total [35S]methionine incorporation during the first few hours after fertilization, there does not appear to be any synthesis of a corresponding M1 subunit (20); if there were one, it would be a very intense band with a molecular mass in the range of 80-90 kD. It therefore seemed likely that the M1 subunit was present as a stockpile of protein in the mature unfertilized oocyte, and that ribonucleotide reductase activity appeared as the newly made M2 subunit combined with the preformed large subunit to generate active ribonucleotide reductase.

In this paper we show that the oocytes do indeed contain a store of the M1 ribonucleotide reductase subunit. It is an 86-kD polypeptide which is already present at its maximum...
level before fertilization. No new synthesis of this subunit is detectable during the first few hours of development, so the appearance of ribonucleotide reductase activity in the zygote is entirely due to activation of translation of the mRNA for the M2 subunit.

Materials and Methods

Buffers

Buffer A: 300 mM glycine, 120 mM glutamic acid, 100 mM Hepes, 10 mM NaCl, 40 mM EDTA, 1 mM MgCl₂, 0.1 mM dithiothreitol, adjusted to pH 7.2 with NaOH.

Buffer B: 100 mM KCl, 50 mM Hepes, 1.1 mM MgCl₂, 0.1 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, adjusted to pH 7.2 with NaOH.

Buffer C: 25 mM Tris, 192 mM glycine, 10% methanol, and 0.1% SDS.

Buffer D: 140 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 8.1 mM Na₂HPO₄, 0.02% sodium azide, and 0.5% Tween 20.

Buffer E: 2 mM urea, 0.1 M NaCl, and 1% Tween 20.

Calcium-free Sea Water: 0.38 M NaCl, 50 mM MgCl₂, 30 mM NaSO₄, 20 mM Hepes, 10 mM KCl, 2 mM NaHCO₃, 1 mM EGTA, pH 7.4, with KOH.

Chemicals and Affinity Columns

The following radiochemicals were purchased from Amersham International (Amersham, UK): [5-³H]-cytidine 5'-diphosphate (CDP) (1 Ci/mg), [5-³H]-CDP-Protein A (affinity purified, 0.1 Ci/mg), and [³H]-thymidine (1,000 Ci/mmol). N-(γ-L-Glutamyl)-L-tyrosine (γ-glu-tyr) and thionine (>1,000 Ci/mmol). N-(γ-L-Glutamyl)-L-tyrosine (γ-glu-tyr) and Ponceau S concentrate were from Sigma Chemical Co. (St. Louis, MO) and polyethyleneimine-cellulose (PEI MN300) thin-layer plates were from Macherey-Nagel (Düren, FRG). All nucleotides were obtained from P-L Biochemicals Inc. (Milwaukee, WI). YL 1/2-Sepharose was a gift from Dr. John Kilmartin (Medical Research Council Laboratory of Molecular Biology, Cambridge). DATP-Sepharose was a gift from Drs. Lars Thelander (Karolinska Institute, Stockholm, Sweden) and B.-M. Sjoberg (University of Uppsala, Sweden).

Clam Extracts

Clams (Spisula solidissima) were collected by the Department of Marine Resources, Marine Biological Laboratory, Woods Hole, MA. They were kept in running sea water at 13-15°C. Oocytes and sperm were isolated as described (20). The oocytes were washed by repeated settling in 0.45-11m pores size nitrocellulose-filtered sea water at 18-20°C, suspended at about 20,000-50,000 cells/ml, and stored at this temperature until required with stirring at 60 rpm. The sperm was stored undiluted at 4°C. To obtain oocyte extracts, the washed cells were allowed to settle and were harvested by a B-4 spin in a bench centrifuge. The cell pellet was washed twice in ice-cold calcium-free sea water and once in ice-cold buffer A. Two volumes of buffer A were then added and the cells homogenized on ice in a metal homogenizer using ~10-15 strokes. Breakage of cells was monitored by light microscopy. The lysate was centrifuged at 12,000 g for 10 min at 4°C and the supernatant was frozen in liquid nitrogen in 1-ml aliquots. Clam oocytes were fertilized with a 20,000-fold dilution of sperm and extracts of embryos were prepared as above. The protein concentration of these postmitochondrial extracts was ~12-14 mg/ml, as determined by the Bradford method (3), using bovine serum albumin as standard.

Preparation of M1 Subunit of Ribonucleotide Reductase

The extract was thawed, mixed with an equal volume of buffer B, and clarified by centrifugation for 5 min in an Eppendorf microfuge. A column of DATP-Sepharose with a vol of 50 μl was prepared in a yellow plastic pipet tip plugged with glass wool. It was equilibrated with 10-15 vol of buffer B at 5°C. A load of 250 μl of a clarified, diluted extract was applied, and the column was then washed with 1 ml of buffer B. The column was then transferred to room temperature (~18°C) and washed with 250 μl of buffer containing 0.25 mM ATP. M1 subunit was eluted with 270 μl of buffer containing 10 mM ATP, the first 20 μl of which were discarded.

1. Abbreviations used in this paper: CDP, cytidine diphosphate; CMP, cytidine monophosphate.

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Table 1. Characterization of Ribonucleotide Reductase from Clam

| pnmol dCDP/ mg per min | % of control |
|-------------------------|-------------|
a. Requirements and inhibitors of embryo activity
| embryo—total mix       | 72.2        | 100.0 |
| -ATP                   | 9.8         | 13.6  |
| Dithiothreitol         | 61.4        | 85.0  |
| -MgCl₂                 | 72.4        | 100.3 |
| -FeCl₂                 | 76.8        | 106.0 |
| +dATP (1 mM)           | 1.3         | 1.8   |
| +Hydroxyurea (1 mM)    | 1.0         | 1.3   |
b. Oocyte activity: effect of 1 mM hydroxyurea
| Extract A              | Control     | 0.6   | 0.9   |
|                        | Hydroxyurea | 0.7   | 1.0   |
| Extract B              | Control     | 1.0   | 1.5   |
|                        | Hydroxyurea | 1.1   | 1.6   |
| Extract C              | Control     | 1.3   | 1.9   |
|                        | Hydroxyurea | 1.1   | 1.6   |

The complete reaction mix contained 0.2 mM [³H]CDP, 5 mM ATP, 8 mM MgCl₂, 5 mM dithiothreitol, 50 μM FeCl₂, 5 mM NaF, and 5 mM Hepes, pH 7.2. Approximately 25 μg of embryo or oocyte extract protein in Buffer B was added in a final volume of 10 μl and the mix incubated for 1 h at 18°C. Ribonucleotide reductase was assayed as described in Materials and Methods.

Milligram amounts of clam M1 for raising antibodies were obtained by several rounds of preparative dATP-Sepharose chromatography, using 5 ml of oocyte extract and 1 ml of dATP-Sepharose each time. The bound protein was eluted with 1 mM dATP in this case, rather than 10 mM ATP. The preparative column was regenerated by washing with 5 mM guanidinium chloride-0.1% (vol/vol) mercaptoethanol, and stored in buffer B containing 0.02% (vol/vol) sodium azide.

Preparation of M2 Subunit of Ribonucleotide Reductase

M2 subunit was prepared by loading 250 μl of a clarified 9-h embryo extract (see above) onto a 50-μl column of monoclonal YL 1/2 antibody-Sepharose extensively pre-washed with buffer B at room temperature. The column was washed with 1 ml of buffer, and the small subunit eluted with a total of 270 μl of buffer containing 5 mM γ-glu-tyr diphosphate. The first 20 μl of the γ-glu-tyr eluate was discarded. Both subunits were thus isolated in buffer at a concentration close to that in the original extract. They were both assayed on the day of isolation. The mini 50-μl columns of YL 1/2-Sepharose and dATP-Sepharose were only used once. It was most important to prewash these columns extensively before use, since 0.02% (vol/vol) sodium azide in the storage buffer was found to completely inhibit ribonucleotide reductase activity.

Ribonucleotide Reductase Assay

All fractions from the affinity columns, including the load sample, were held at room temperature until the small subunit had been eluted, at which point they were placed on ice. The assay volume was 10 μl, 2 μl of which consisted of a master mix containing 1.0 mM [5-³H]-CDP (specific activity of 70,000-800,000 cpm/nmol), 25 mM ATP, 40 mM MgCl₂, 25 mM dithiothreitol, 250 μM FeCl₂, 25 mM NaF, and 25 mM Hepes, pH 7.2. The volume of column fractions tested was 4 μl. Since the presumptive large subunit fractions contained 10 mM ATP, assays containing these fractions used a master mix which contained one-fifth the usual amount of ATP to give a final concentration of 5 mM. Duplicate samples were incubated for 1 h in a water bath at 18°C and the reaction was terminated by the addition of 10 μl of 2 M perchloric acid. Cytidine monophosphate (CMP) and dCMP standards were added to a final concentration of 2 μM each and the tubes were boiled for 10 min to hydrolyze the substrate and product to their monophosphate forms. The samples were neutralized with 4 M KOH using phenol red as indicator. The precipitate of K perchlorate was removed by centrifugation in an Eppendorf microfuge and 10 μl of the supernatant applied to polyethyleneimine-cellulose thin-layer plates. Samples were applied in broad streaks ~1 cm wide, and then allowed to dry. The plates were first
developed in water, up to a height of 5 cm, and then dried before being developed in 3% boric acid in 30 mM LiCl (4–5 cm) followed by 3% boric acid in 0.6 M LiCl (a further 8–10 cm; reference 29). Typical Rf values for CMP and dCMP were 0.15 and 0.29, respectively. dCMP spots, visualized under UV, were cut out, dried, and counted in 0.5% 2,5-diphenyloxazole in toluene. Fluorography of duplicate plates, by immersion in 0.4% 2,5-diphenyloxazole in molten naphthalene (1) showed that only two spots, corresponding to CMP and dCMP, were obtained. The rate of formation of dCMP was linear for 60 min, and linear with respect to the value of added extract.

Preparation of Anti-M1 Antibody

A total of ~1.0 mg of presumptive M1 was isolated by preparative affinity chromatography of clam oocyte extract on dATP-Sepharose, followed by gel electrophoresis in 3-mm thick SDS polyacrylamide gels. The gels were washed in cold water to remove excess SDS and soaked in cold 0.25 M KCl. The opaque M1 band was excised from the gels, homogenized with water by successive passages through syringes of diminishing diameter, and injected subcutaneously in 4–5 sites in the back of a rabbit. In all, three injections were given, on days 1, 17, and 43, with ~350 μg each of protein. A preimmune bleed was obtained on day 1 and the immune bleed on day 52. After overnight coagulation of the blood, serum was collected by centrifugation, supplemented with 0.02% sodium azide, and stored at ~80°C. Sera were tested by Western blotting as described below. The day-52 bleed contained antibodies reacting with the antigen, whereas no such antibodies could be detected in preimmune serum (data not shown).

Western Blots

SDS polyacrylamide gels were run as previously described (23). Gels were assembled in a sandwich, against nitrocellulose filter paper and surrounded by Whatman filter paper sheets, and placed in a transfer tank containing buffer C. Quantitative transfer took place during 90 min at 0.5 A at room temperature. The opaque M1 band was excised from the gels, homogenized with water by successive passages through syringes of diminishing diameter, and injected subcutaneously in 4–5 sites in the back of a rabbit. In all, three injections were given, on days 1, 17, and 43, with ~350 μg each of protein. A preimmune bleed was obtained on day 1 and the immune bleed on day 52. After overnight coagulation of the blood, serum was collected by centrifugation, supplemented with 0.02% sodium azide, and stored at ~80°C. Sera were tested by Western blotting as described below. The day-52 bleed contained antibodies reacting with the antigen, whereas no such antibodies could be detected in preimmune serum (data not shown).

Results

Characterization of Clam Ribonucleotide Reductase

We previously found that there is no detectable synthesis of the small subunit of ribonucleotide reductase in clam oocytes and that it begins to be made at a high rate shortly after fertilization (20, 21). We therefore assayed ribonucleotide reductase enzymic activity in oocytes and embryos at various stages of development, and characterized the enzyme in terms of its cofactor requirements and subunit composition. Ribonucleotide reductase activity was measured directly in postmitochondrial extracts. The extracts were supplemented with 0.2 mM [3H]CDP, 5 mM ATP, 8 mM MgCl2, 5 mM dithiothreitol, 50 mM FeCl3, 5 mM NaF, and 5 mM Hepes, pH 7.2 (final concentrations) and incubated for 1 h at 18°C. The conversion of [3H]CDP to [3H]dCDP was determined by hydrolysis to the mononucleotide and chromatography on polyethylenimine-cellulose as described in Materials and Methods. Initial experiments indicated that maximum levels of reductase activity were obtained in embryos 6–9 h after fertilization (data not shown). As shown in Table I a, this maximum level corresponded to ~70 U/mg extract protein (1 U = 1 pmol dCDP formed per minute). Addition of ATP to the extracts was required for CDP reduction; only 13% of the control value was obtained in its absence (Table I a). Although we routinely added dithiothreitol, Mg2+, and Fe3+ to the assay mix, their omission from the buffer had little or no effect on the measured levels of activity. This result indicates that sufficient levels of these cofactors, or their biological equivalents in the case of dithiothreitol, are present in the extracts. The activity was inhibited to 1–2% of the control value by addition of 1 mM dATP or 1 mM hydroxyurea (Table I a). dATP is a classic allosteric inhibitor of this enzyme (8), and hydroxyurea destroys the free radical of all iron-containing ribonucleotide reductases (24). Thus clam ribonucleotide reductase has the same general properties as the well-characterized two-subunit calf thymus enzyme (8), and is very unlikely to be a vitamin B12–dependent single-subunit type of enzyme such as found in Lactobacillus or Euglena (24).

Ribonucleotide Reductase Activity is Only Detectable after Fertilization of Clam Oocytes

Oocyte extracts appeared to contain ~1% of the ribonucleotide reductase activity of the 9-h (~80-cell) gastrula extracts (Table I b). This low level is reproducibly above the background measured with buffer B in place of the oocyte extract; however, it was not inhibited by hydroxyurea (Table I b), and probably represents the true background of our assays. Consistent with this interpretation is the finding that ribonucleotide reductase in active embryo extracts was not inhibited below this level by either hydroxyurea or dATP (Table I a). The lack of oocyte activity was not ascribable to the presence of an inhibitor of ribonucleotide reductase, as shown by mixing experiments (data not shown). Thus we conclude that oocytes do not possess measurable ribonucleotide reductase activity.

Preparation of the Small Subunit of Ribonucleotide Reductase by Immunoaffinity Chromatography

The small subunit of ribonucleotide reductase binds tightly to Sepharose 4B coupled to the anti-α-tubulin monoclonal antibody, YL 1/2 (13, 23). This is almost certainly due to the homology between the COOH-terminal residues of ribonucleotide reductase M2 subunit (asp-alasasp-phe) and the COOH-terminus of α-tubulin (gly-glu-glu-tyr) (23). Antibody YL 1/2 does not bind α-tubulin lacking its COOH-terminal tyrosine residue (27), and detailed studies of the specificity of this antibody have shown that it recognizes the COOH-terminal residues of α-tubulin. The binding of tubulin to the antibody can be quite effectively prevented even by the dipeptide asp-phe, though longer model peptides display a higher affinity (26). In view of this we thought it should be possible to elute the bound M2 polypeptide from the antibody affinity column under very mild conditions using the dipeptide γ-glu-tyr, which Wehland et al. (26) had characterized as an effective competitor of tubulin binding to YL 1/2 and which is commercially available. We should thus be able to obtain pure enzymically active M2 subunit to use in reconstitution assays to detect the presence of the large (M1) subunit of ribonucleotide reductase.

To test whether γ-glu-tyr could be used to purify M2 in this way, a cell-free homogenate was made from embryos labeled with [35S]methionine for 3 h after fertilization. Two aliquots of this extract were passed over two separate YL
Figure 1. Isolation of the small subunit of ribonucleotide reductase by affinity chromatography on YL 1/2-Sepharose: elution with γ-glut-tyr. (a) A [35S]methionine-labeled clam embryo extract was fractionated on two YL 1/2-Sepharose columns. (Lane 1) Load; (lane 2) flow-through. The two columns were washed with buffer B containing increasing concentrations of KCl, in the presence (+) and absence (−) of 0.5 mM γ-glut-tyr. (Lanes 3 and 4) 0.1 M; (lanes 5 and 6) 0.3 M; (lanes 7 and 8) 0.5 M; (lanes 9 and 10) 0.8 M; (lanes 11 and 12) 1.0 M; (lane 13) 1.5 M. Finally, the columns were washed with SDS gel sample buffer (lanes 14 and 15). Samples of each fraction were analyzed by SDS PAGE and the gel was autoradiographed. (b) A column of YL 1/2-Sepharose, loaded with a labeled extract as in Fig. 1 a, was washed with 0.1 M KCl-buffer B containing increasing concentrations of γ-glut-tyr. (Lane 1) 0.5 mM; (lane 2) 1.0 mM; (lane 3) 1.5 mM; (lane 4) 2.0 mM; (lane 5) 2.5 mM; (lane 6) 5.0 mM. The last two washes were in 0.2 M KCl-buffer B containing 1.5 mM γ-glut-tyr (lane 7) and 2.0 mM γ-glut-tyr (lane 8).

1/2-Sepharose CL-4B columns, one of which was eluted with buffer containing increasing concentrations of KCl, while the second column was eluted with the same buffers supplemented with 0.5 mM γ-glut-tyr. As noted earlier (23) and shown in Fig. 1 a (compare lanes 1 and 2), the M2 subunit (p42) was completely removed from the extract by passage over the YL 1/2 column. Salt elution of M2 in the absence of γ-glut-tyr began at 0.8 M KCl and continued at 1.0 M and 1.5 KCl (Fig. 1 a, lanes 9, 11, and 13). However, a substantial amount of p42 still remained on the column, and could only be eluted with SDS gel sample buffer (lane 14). On the other hand, in the presence of 0.5 mM γ-glut-tyr, elution of p42 was essentially complete at 0.5 M KCl (lane 8), and very little remained bound to the column after the most stringent wash (lane 15). Since we wished to assay ribonucleotide reductase activity, and 0.5 M KCl is too high a salt concentration for the enzyme assay, a similar experiment was performed holding the KCl concentration at 0.1 and 0.2 M and increasing the concentration of γ-glut-tyr. Quantitative elution was achieved with 5 mM γ-glut-tyr at 0.1 M KCl (Fig. 1 b, lane 6). When the Coomassie Blue–stained gels corresponding to these autoradiographs were examined, it was observed that both α- and β-tubulin co-eluted with the small subunit of ribonucleotide reductase under all conditions tested (not shown, but see Fig. 3 a).

Table II. Purification of the Small Subunit of Ribonucleotide Reductase by Affinity Chromatography on YL 1/2-Sepharose

|                         | pmol dCDP/   | % of control |
|-------------------------|--------------|--------------|
|                         | mg per min   |
| a. Ribonucleotide reductase activity of embryo extracts and fractions from YL 1/2-Sepharose |             |
| Embryo load             | 39.4         | 100.0        |
| Flow-through            | 1.0          | 2.5          |
| γ-Glu-tyr eluate        | 0.5          | 1.3          |
| Flow-through + eluate   | 34.0         | 86.3         |
| b. Activation of cryptic ribonucleotide reductase activity in crude oocyte extracts by addition of affinity-purified small subunit |             |
| Oocyte                  | 0.6          | 1.5          |
| Oocyte + embryo γ-glut-tyr eluate | 29.8 | 75.6         |
| Oocyte + oocyte γ-glut-tyr eluate* | 0.4 | 1.0          |

A postmitochondrial embryo extract was fractionated on YL 1/2-Sepharose. Bound protein was eluted with γ-glut-tyr. The load, flow-through and γ-glut-tyr fractions were assayed for ribonucleotide reductase as described in Materials and Methods. The activity of the γ-glut-tyr eluate was expressed per mg extract protein.

* For this control experiment, an oocyte extract was fractionated on YL 1/2-Sepharose.
Figure 2. Purification of the large subunit of ribonucleotide reductase by affinity chromatography on dATP-Sepharose. (a) A post-mitochondrial oocyte extract was fractionated on dATP-Sepharose as described in Materials and Methods. After several column washes with buffer B, the column was washed with buffer B containing 0.25, 10, and 20 mM ATP and finally 1 mM dATP. Protein was only obtained with the 10 mM ATP wash (not shown). Equal aliquots of fractions were analyzed on an SDS polyacrylamide gel which was stained with Coomassie Blue. (Lane 1) Load; (lane 2) flow-through; (lane 3) 10 mM ATP eluate; (lane 4) as lane 3 but 10-fold protein load. (b) A duplicate gel to that shown in Fig. 2a was transferred to nitrocellulose and incubated with rabbit anti-clam 86-kD protein antibodies (see Materials and Methods) followed by [\( ^{125} \)I]protein A. Shown here is the portion of the blot corresponding to the antigen. Lanes 1'-4' are equivalent to lanes 1-4 in Fig. 2a.

The small subunit as isolated under these conditions retained its activity. 250 μl of a 9-h embryo extract was loaded onto a 50 μl YL 1/2-Sepharose column. The flow through was collected, and the column was washed with 1 ml of buffer B before elution with 250 μl of 5 mM γ-glut-tyr in buffer B. The γ-glut-tyr eluted fraction should thus contain the same concentration of the small subunit as the initial load sample. The YL 1/2 column removed >97% of the ribonucleotide reductase activity from the loaded extract (Table IIa), and the γ-glut-tyr eluate was also inactive. However, >85% of the initial activity was reconstituted when the γ-glut-tyr eluate was mixed with the flow-through fraction (Table IIa). This result indicated that the small subunit was active and that another component, most likely the M1 subunit, was also required for clam reductase activity.

The Small Subunit of Ribonucleotide Reductase Isolated from Embryos Confers Activity on Oocyte Extracts

The next question was whether the M1 subunit is present in the oocyte before fertilization. If it is, addition of M2 subunit to an oocyte extract should generate reductase activity. When an affinity column--purified preparation of small subunit from embryos was added to an oocyte extract, ribonucleotide reductase activity at ~75% of the maximal embryo level was observed (Table IIb). Control experiments showed that a γ-glut-tyr eluate obtained from passing oocyte extracts over the YL 1/2 column did not confer activity on a total oocyte extract (Table IIb). Although these oocyte γ-glut-tyr eluates lacked p42, they did contain both α- and β-tubulin, showing that the column had worked properly (data not shown). Thus the oocyte extract contains a cryptic component of ribonucleotide reductase, in amounts sufficient to generate nearly the same activity as is found in late embryos.

Isolation of the Large Subunit of Ribonucleotide Reductase by Affinity Chromatography on dATP-Sepharose

Purification of the large subunit of ribonucleotide reductase from mammalian sources has generally been accomplished by affinity chromatography on dATP-Sepharose (8, 24). It is thought that the tight binding of the 88-kD M1 polypeptide to this matrix occurs via its regulatory nucleotide binding site. The M1 polypeptide can be eluted with 1 mM dATP, or by higher concentrations (10–50 mM) of ATP for which the enzyme has a much lower affinity (8). Since dATP is an inhibitor of ribonucleotide reductase whereas ATP is an essential cofactor, the column is usually eluted with ATP when enzymically active M1 is required.

Passage of a 9-h clam embryo extract over dATP-Sepharose removed 98% of ribonucleotide reductase activity, whereas unmodified Sepharose did not bind measurable enzymic activity (data not shown). To see whether dATP-Sepharose bound a polypeptide of appropriate size (~80–90 kD), an oocyte extract was passed over a column of this affinity matrix. After several column washes with 0.1 M KCl-containing buffer, bound proteins were eluted in buffer containing 0.25, 10, and 20 mM ATP and finally with 1 mM dATP. As shown in the Coomassie Blue-stained gel in Fig. 2a, a single major protein of 86 kD was eluted with 10 mM ATP (lane 3) and further ATP or dATP washes did not increase its yield (data not shown). Thus, by virtue of its binding to this column and its size, this protein was a likely candidate for the large subunit. The flow-through from dATP-Sepharose did not lack a major staining band at 86 kD. However quantitation by scanning densitometry of the 86-kD protein

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eluted by 10 mM ATP suggested that it comprised 0.4% of postmitochondrial oocyte extract protein. This value is similar to that of the two tubulin chains, as isolated by YL 1/2-Sepharose chromatography, which each constitute ~0.5% of total protein in our extracts as estimated by densitometry of the Coomassie-stained proteins.

The dATP-Sepharose was used to purify large amounts of p86 in order to obtain polyclonal antibodies against it. As described in Materials and Methods, we obtained reasonably high-titer serum that specifically reacted with p86. This antibody was used to show that the 86-kD polypeptide was quantitatively bound by dATP-Sepharose and completely eluted with 10 mM ATP. A duplicate of the gel shown in Fig. 2 a was transferred to nitrocellulose and incubated with rabbit anti-clam 86-kD protein antibodies followed by [125I]-protein A. Shown here is the portion of the blot corresponding to the antigen. Lanes 1'-6' are equivalent to lanes 1-6 in Fig. 3 a.

The radioactive band is also ~10-fold higher (Fig. 2 b, lane 4'), showing that the blot was incubated with excess antibody and protein A. Thus we concluded that the presumptive M1 subunit of ribonucleotide reductase was completely bound and quantitatively recovered by our procedures.

The Isolated M1 and M2 Subunits Are Each Inactive Alone, but Together They Generate Ribonucleotide Reductase Activity

We were now in a position to test whether the p86 eluted from dATP Sepharose was the M1 subunit of clam ribonucleotide reductase, by mixing it with the γ-glut-tyr eluate from the YL 1/2-Sepharose affinity matrix. A Coomassie Blue-stained gel of the load (lanes 1 and 4), flow-through (lanes 2 and 5), and bound fractions from both columns is shown in Fig. 3 a. The oocyte ATP eluate contained a major polypeptide of 86 kD (lane 3). When an equivalent amount (on a cell basis) of the embryo γ-glut-tyr fraction was analyzed, only the tubulin polypeptides were detected by Coomassie staining (Fig. 3 a, lane 6). The 42-kD subunit of ribonucleotide reductase was only ap-
Activity from Isolated Subunits

| Isolated fractions          | pmol dCDP/mg per min | % of control |
|----------------------------|----------------------|--------------|
| YL 1/2-Sepharose            |                      |              |
| Embryo                     | 34.5                 | 100          |
| Flow-through                | 0.0                  | 0            |
| γ-Glu-tyr eluate            | 0.7                  | 2            |
| dATP-Sepharose              |                      |              |
| Oocyte                     | 0.0                  | 0            |
| Flow-through                | 0.6                  | 2            |
| ATP eluate                  | 1.1                  | 3            |
| Mixed fractions             |                      |              |
| γ-Glu-tyr eluate + ATP eluate | 10.7                | 31           |
| γ-Glu-tyr eluate + ATP buffer | 0.0                  | 0            |
| γ-Glu-tyr buffer + ATP eluate | 0.7                  | 2            |

The two purified fractions were assayed for ribonucleotide reductase activity. The results obtained from this experiment are given in Table III. Both subunits were inactive when assayed alone. The activity of the small subunit was checked by recombining it with the flow-through fraction obtained from the embryo-YL 1/2 column; 92% of original activity was reconstituted (data not shown). When the M2 subunit was mixed with the presumptive M1 subunit up to 31% of the embryo activity was reconstituted. The extent of reconstitution in three different experiments varied from 25–52%. No activity was obtained by mixing the small subunit with the ATP buffer used to elute the large subunit, or the large subunit with the γ-glut-tyr buffer. We conclude from these experiments that the 86-kD polypeptide purified from oocyte extracts by dATP-Sepharose chromatography is indeed the large subunit of ribonucleotide reductase.

The two subunits of clam ribonucleotide reductase evidently have very low affinity for each other, because as Fig. 3 b shows, the YL 1/2 matrix does not bind measurable amounts of M1 subunit, although it removes essentially all the small subunit from crude extracts.

The Level of the Large Subunit Is Constant during Early Development

The immunoblot of clam extracts probed with serum raised against the large subunit shown in Fig. 3 b reveals that the amount of M1 is the same in the oocyte and in the 9-h embryo (lanes 1' and 4'). Furthermore, no increase in M1 levels was observed when total proteins from oocytes and 24-h embryos were similarly analyzed by the Western blot technique (data not shown). Since the immunoblots were carried out with excess antibody and with excess iodinated protein A (see Fig. 2 b), these data show that the mature clam oocyte stockpiles a sufficient concentration of the large subunit of ribonucleotide reductase to support multiple rounds of DNA replication during the first day of development (see Discussion).

Discussion

This paper describes the identification of the large (M1) subunit of clam ribonucleotide reductase as an entity present in unfertilized oocytes which complements partially purified M2 subunit to yield active enzyme. This complementing activity belongs to a polypeptide of 86 kD that binds to dATP-Sepharose. Thus the molluscan enzyme appears to be very similar to the well-characterized mammalian ribonucleotide reductase, as might be expected from the high homology shown between the amino acid sequences of the clam and mouse M2 subunits (80–90%; L. Thelander, personal communication). The concentration of M1 is high in the oocyte, and stays constant for at least 24 h after fertilization. During this period, ribonucleotide reductase activity rises from a barely detectable baseline to levels sufficient to provide the deoxyribonucleotide precursors needed for the rapid DNA synthesis that occurs during cleavage. The increase in ribonucleotide reductase activity seems to be entirely due to an increase in the concentration of the M2 subunit provided by de novo protein synthesis programmed by maternal mRNA. This mRNA is stored in the oocyte in untranslated form, and it is rapidly loaded onto polysomes shortly after fertilization (20, 21). At the same time, its 3' terminus is elongated by polyadenylation (21). By contrast, little or no synthesis of M1 occurs during cleavage, judged by the constancy of its level and our failure to find significant amounts of dATP-binding [35S]methionine-labeled p86 (our unpublished experiments).

We could not detect ribonucleotide reductase activity in clam oocytes. This seems to be a rather variable feature in other invertebrate eggs. Noronha et al. (17) also could not detect activity in the eggs of the sea urchin Arbacia punctulata. De Petrocellis and Rossi (6) obtained the same result with eggs of a related urchin, Arbacia lixula, but found that Paracentrotus lividus eggs contained ~10–20% of maximum embryo activity. In both these studies, the embryo activities were determined to be 1–5 pmol CDP reduced/mg protein per min. The maximal activity we obtained was 10–50 times higher than this in both clams and in the sea urchin, Arbacia punctulata (this paper and reference 23). Despite the improved sensitivity of our assay procedure, we were unable to detect ribonucleotide reductase activity in clam oocytes.

Indirect evidence suggests that <1 genome (1.2 pg) equivalent of dNTPs has accumulated during clam oogenesis. When hydroxyurea, a specific inhibitor of ribonucleotide reductase, is added just after fertilization, it inhibits the first cell division (data not shown). Similar experiments and direct analysis indicate that the store of dNTPs in urchin eggs is larger than this, but not much (2, 14). The origin of these stores is not known. Thus, both these marine invertebrates depend on ribonucleotide reductase to supply the precursors required for the early embryonic cell divisions which occur at 30–60-min intervals for the first 6 or so hours after fertilization.

Other enzymes involved in the system of ribonucleotide reduction do not show any increase in activity at fertilization. We measured two enzymes that are probably involved in producing the reducing power used by clam ribonucleotide reductase. Glucose-6-phosphate dehydrogenase reduces NADP to NADPH, and has the same activity in extracts of both fertilized and unfertilized oocytes. The thioredoxin system, comprising both thioredoxin and thioredoxin reductase, was assayed by the insulin reduction test (12) and showed no increase in activity between oocyte and embryo extracts (data not shown). These findings conform with previous observations showing no changes at or within a few hours of fertiliza-
tion in the activities of DNA polymerase, thymidine kinase, thymidylate synthetase, or dCMP deaminase in a variety of species of sea urchins (15, 16, 22).

It is interesting to compare the regulation of the activity of clam ribonucleotide reductase in early development with its cell cycle regulation found in exponentially growing mammalian cells. As shown by Thelander and co-workers, ribonucleotide reductase activity rises and falls during the cell cycle in cultured mouse cells, peaking in S phase (7, 9). As in the case of clam embryos, these variations in activity appear to be mediated by variations in the level of the small subunit (7, 9). As far as we know, no such cell-cycle variation in enzyme levels are found in clams or sea urchins, but this is hardly surprising in view of the length of the cycles; indeed, the total length of the period of cleavage is comparable to a single mammalian cell's S-phase! At the end of cleavage, the small subunit mRNA declines quite rapidly in level (5, 19).

The unusual mode of enzyme regulation during early embryonic development that we have described is, as far as we are aware, unique to ribonucleotide reductase. The small subunit of ribonucleotide reductase belongs to the class of proteins whose synthesis at a high rate is confined to the rapid cleavage stage of clam and sea urchin development (10, 28). For some reason its presence in the oocyte is undesirable: it may either be unstable so that its storage would be impossible, or else its presence may be incompatible with the needs of a nondividing cell. On the other hand, the large subunit of ribonucleotide reductase is apparently a very stable protein which is stored during oogenesis. It would not be surprising if other examples of this sort were to be found.

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References

1. Bonner, W. M., and J. D. Stedman. 1978. Efficient fluorography of 'H and 14C on thin layers. Anal. Biochem. 89:247-256.
2. Brachet, J. 1967. Effects of hydroxyurea on development and regeneration. Nature (Lond.) 214:1122-1133.
3. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72:248-254.
4. Broadhurst, B. 1984. The informational content of the echinoderm egg. In Developmental Biology: A Comprehensive Synthesis. I. Oogenesis. L. Browder, editor. Plenum Publishing Corp., New York. 525-576.
5. Bray, S., and T. Hunt. 1983. Developmental studies of a major maternal mRNA in Arbacia punctulata. Biol. Bull. 165:499-500.
6. De Petrocellis, B., and M. Rossi. 1976. Enzymes of DNA biosynthesis in developing sea urchins. Dev. Biol. 48:250-257.
7. Engström, Y., S. Eriksson, L. Jildevik, S. Skog, L. Thelander, and B. Tribukait. 1985. Cell cycle-dependent expression of mammalian ribonucleotide reductase. J. Biol. Chem. 260:9141-9146.
8. Engström, Y., S. Eriksson, L. Thelander, and M. Åkerman. 1979. Ribonucleotide reductase from calf thymus. Purification and properties. Biochemistry. 18:2941-2948.
9. Eriksson, S., A. Gräslund, S. Skog, L. Thelander, and B. Tribukait. 1984. Cell cycle-dependent regulation of mammalian ribonucleotide reductase. J. Biol. Chem. 259:11695-11700.
10. Grainger, J. L., A. von Brun, and M. M. Winkler. 1986. Transient synthesis of a specific set of proteins during the rapid cleavage phase of sea urchin development. Dev. Biol. 114:27-28.
11. Hultin, T. 1961. The effect of paromycin on protein metabolism and cell division in fertilized sea urchin eggs. Experientia. 17:410-411.
12. Jackson, R. J., P. Herbert, E. A. Campbell, and T. Hunt. 1983. The role of sugar phosphates and deoxyribonucleotidyl synthetases in the control of reticulocyte protein synthesis. Eur. J. Biochem. 131:313-324.
13. Kilmartin, J. V., B. Wright, and C. Milstein. 1982. Rat monoclonal antitubulin antibodies derived by using a new nonsecreting rat cell line. J. Cell Biol. 94:576-582.
14. Matthews, C. K. 1975. Giant pools of DNA precursors in sea urchin eggs. Exp. Cell Res. 92:47-56.
15. Morris, P. W., and W. J. Rutter. Nucleic acid polymerizing enzymes in developing Strongylocentrotus franciscanus embryos. Biochemistry. 15:3106-3113.
16. Nagano, H., and Y. Mano. 1968. Thymidine kinase, thymidylate kinase and 32P and 3H thymidine incorporation into DNA during early embryogeny of the sea urchin. Biochim. Biophys. Acta. 157:546-557.
17. Noronha, J. M., G. H. Sheys, and M. J. Buchanan. 1972. Induction of a reductive pathway for deoxyribonucleotide synthesis during early embryogenesis of the sea urchin. Proc. Natl. Acad. Sci. USA. 69:2006-2010.
18. Raff, R. A., and R. Showman. 1983. Maternal messenger RNA. In The Cell Cycle of Fertilization. C. B. Metz and A. Monroy, editors. Academic Press, Inc., New York. 401-452.
19. Rosenthal, E. T. 1983. Translational control of gene expression during early development. Ph.D. thesis. Harvard University. 192 pp.
20. Rosenthal, E. T., T. Hunt, and J. V. Ruderman. 1980. Selective translation of mRNA controls the pattern of protein synthesis during early development of the surf clam, Spisula solidissima. Cell. 20:487-496.
21. Rosenthal, E. T., T. R. Tansey, and J. V. Ruderman. 1983. Sequence-specific depletions and depletions for changes in the translation of maternal mRNA after fertilization of Spisula oocytes. J. Mol. Biol. 166:309-327.
22. Scarano, E., and R. Maggio. 1959. The enzymatic deamination of 5'-deoxyadenosine and of 5-methyl-5'-deoxycytidylic acid in the developing sea urchin embryo. Exp. Cell Res. 18:333-346.
23. Standart, N. M., S. J. Bray, E. L. George, T. Hunt, and J. V. Ruderman. 1985. The small subunit of ribonucleotide reductase is encoded by one of the most abundant translationally regulated maternal mRNAs in clam and sea urchin eggs. J. Cell Biol. 100:1968-1976.
24. Thelander, L., and P. Reichard. 1979. Reduction of ribonucleotides. Annu. Rev. Biochem. 48:133-158.
25. Wagenaar, E. B. 1983. The timing of synthesis of proteins required for mitosis in the cell cycle of the sea urchin embryo. Exp. Cell Res. 144:393-403.
26. Wehland, J., H. C. Schroeder, and K. Weber. 1984. Amino acid sequence requirements in the epitope recognized by the a-tubulin-specific rat monoclonal antibody YL I/2. Eur. J. Biochem. 157:11695-11700.
27. Wehland, J., M. C. Willingham, and I. V. Sandoval. 1983. Amino acid sequence requirements in the epitope recognized by the a-tubulin-specific rat monoclonal antibody YL I/2. Exp. Cell Res. 93:576-582.
28. Yeh, Y.-C. 1978. A simple and sensitive assay procedure for ribonucleotide reductase system. Anal. Biochem. 86:175-183.
29. Young, C. W., J. J. Hendler, and D. A. Karnofsky. 1969. Synthesis of protein for DNA replication and cleavage events in the sand dollar embryo. Exp. Cell Res. 58:15-26.