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Identification of the mRNA and polypeptide subunit for prostaglandin endoperoxide synthase from mouse mastocytoma P-815 cells

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Cloned mouse mastocytoma P-815,2-E-6 cells are barely able to synthesize prostaglandins because of a lack of prostaglandin endoperoxide synthase activity. However, the addition of sodium *n*-butyrate at 1 mM induces synthesis *de novo* of prostaglandins in this cell line. Employing this system, we could isolate an mRNA for prostaglandin endoperoxide synthase by a combination of cell-free translation and immunoprecipitation. The antibody, prepared in rabbit by injecting purified prostaglandin endoperoxide synthase from bovine vesicular gland, was shown to cross-react with the corresponding enzyme from 2-E-6 cells. The poly(A)-containing mRNA has a sedimentation coefficient of 17S and codes for a single polypeptide chain of M_r 62000 as estimated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The M_r of the mouse polypeptide chain appears very similar to that of the purified carbohydrate-free prostaglandin endoperoxide synthase from sheep vesicular gland. These findings are a contribution to the isolation of the gene for prostaglandin endoperoxide synthase.

Prostaglandin endoperoxide synthase (PES; EC 1.14.99.1) exhibits both cyclo-oxygenase and hydroperoxidase activities and thus is capable of carrying out the first two reactions in prostaglandin biosynthesis. The M_r of PES in the native state in the presence of non-ionic detergent has been estimated at 125000–145000 and 300000–350000 for sheep and cow, respectively (Miyamoto *et al.*, 1976; Roth *et al.*, 1980; Van der Ouderaa *et al.*, 1977), indicating an oligomeric structure. To date, however, no conclusive evidence has been presented whether identical or distinct subunits are involved in the multimeric form of PES.

In cloned mouse mastocytoma P-815,2-E-6 cells, PES activity can be induced by 1 mM-sodium *n*-butyrate (Koshihara *et al.*, 1980, 1981*b*). We have isolated the poly(A)-containing mRNA coding for PES from the treated cells, which was identified by cell-free translation followed by immunoprecipitation. The results are discussed in terms of the subunit structure of mouse PES.

Abbreviations used: PES, prostaglandin endoperoxide synthase; SDS, sodium dodecyl sulphate.

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Experimental

Materials

Cloned mastocytoma P-815,2-E-6 cells were cultured in suspension in Eagle's basal medium supplemented with 10% (v/v) foetal bovine serum under CO_2/air (1:19) at 37°C using a gyrotory shaker (model G2, New Brunswick Scientific Co.) as described previously (Kawamura *et al.*, 1980). Their generation time was about 13–14h. Cells in the exponential growth phase were treated with 1 mM-sodium *n*-butyrate for 26h in order to induce the mRNA for PES (Koshihara *et al.*, 1981*b*). Then cells were harvested by centrifugation, washed with phosphate-buffered saline (Ca^{2+}- and Mg^{2+}-free) and stored at −80°C until use.

Preparation of poly(A)-containing mRNA

Poly(A)-containing mRNA was prepared from *n*-butyrate-treated cells according to the method of Krystosek *et al.* (1975) with a slight modification. In brief, cells (4.9×10^9) were suspended in 20ml of homogenizing buffer consisting of 50mM-Tris/HCl (pH 7.5), 5mM-$MgCl_2$, 40mM-NaCl and heparin (0.5mg/ml), and homogenized with a Dounce homogenizer. The homogenate was centrifuged at 700*g* for 10min in order to remove nuclei. The nuclear fraction was washed once with homo-

genizing buffer by centrifugation. Then both supernatant fractions were centrifuged at 10000*g* for 25min, and the resulting supernatant was re-centrifuged at 272000*g* for 50min. Polysomes precipitated were suspended in 0.5M-NaCl containing 0.5% SDS at a concentration of $25A_{260}$/ml, and poly(A)-containing mRNA was prepared by oligo(dT)–cellulose column chromatography. The column size was 2.5ml and the flow rate was 0.6ml/min. Poly(A)-containing mRNA was eluted with water.

The poly(A)-containing mRNA was further fractionated by a 10–25% (w/v) linear sucrose density gradient containing 10mM-Tris/HCl, pH7.5, 100mM-NaCl and 1mM-EDTA in a Beckman SW 27 rotor at 95000*g* for 18h. Poly(A)-containing mRNA in each fraction (1ml) was concentrated by ethanol in the presence of 20μg of purified *Escherichia coli* tRNA/ml.

Cell-free translation and immunoprecipitation

The template activity of poly(A)-containing mRNA was determined by incorporation of [^{35}S]-methionine (sp. radioactivity 1420Ci/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.) into protein in a cell-free system from rabbit reticulocytes as described by Pelham & Jackson (1976). RNA and [^{35}S]methionine were added at concentrations of 1.5–3.0μg and 11μCi per 11μl, respectively. The assay mixture was incubated at 30°C for 60min. These conditions of RNA contents and incubation period were optimum for the assay. The product of translation *in vitro* before and after immunoprecipitation was analysed by 8.75% SDS/polyacrylamide-gel electrophoresis by the method of Laemmli (1970). Immunoprecipitation was facilitated by the addition of *Staphylococcus aureus* protein A. After gel electrophoresis ^{35}S-labelled translation products were detected by fluorography.

The antibody was prepared from antiserum of rabbit immunized with PES purified from bovine vesicular gland according to Smith & Wilkin (1977), and was capable of cross-reacting with the PES from cultured mouse mastocytoma cells (see Fig. 2).

Results and discussion

Template activity of poly(A)-containing mRNA fractionated by sucrose gradient

We obtained about 1.6mg of crude poly(A)-containing RNA from 4.9×10^9 *n*-butyrate-induced cells after oligo(dT)–cellulose chromatography as described in the Experimental section. This RNA was fractionated by a 10–15% linear sucrose gradient. The distribution of RNA across the gradient indicates no particular concentration of RNA at a specific size region, except for the fractions near the bottom, as well as a minimal contamination of rRNA. The RNA in the bottom fractions is virtually devoid of template activity in a cell-free system.

The polypeptides synthesized *in vitro* on each sucrose fraction were analysed by SDS/polyacrylamide-gel electrophoresis. The polypeptide pattern was almost identical for all fractions except that lower molecular weight polypeptides predominated in fractions after fraction 25, corresponding to a template size below 12S. Major polypeptides have apparent M_r values of 55000, 62000, 67000, 75000 and 81000, respectively (Fig. 1*b*). It is noteworthy that a polypeptide of M_r 20000 was a major product of translation of the RNA from fraction 27 (10–11S).

Identification of the PES peptide of the translation product in vitro by immunoprecipitation

We have previously purified PES from bovine vesicular gland to homogeneity (Koshihara *et al.*, 1981*a*). Antibody was raised in rabbits against the pure bovine enzyme and partially purified. The double immunodiffusion test demonstrated that this antibody could specifically cross-react with the PES fraction prepared from induced mouse cells (Fig. 2). It did not react with the prostaglandin isomerase fractions of mouse mastocytoma cells nor with bovine serum albumin (Fig. 2). Based on this fact, we attempted to identify a specific polypeptide(s) by immunoprecipitation of those synthesized in a cell-free system. As shown in Fig. 1(*a*), the polypeptide(s) synthesized in response to the fraction 20 RNA was more specifically immunoprecipitated. SDS/polyacrylamide-gel electrophoretic analysis confirmed this observation (Fig. 1*c*), in which one single polypeptide of M_r 62000 was clearly demonstrated by mRNA from fractions 21 and 22. A higher radioactivity ratio than expected of the immunoprecipitate from the fraction 20 to total products (approx. 18%) was due to nonspecific binding of free [^{35}S]methionine to translation products, as judged by a high general background (10%) expressed as the radioactivity ratio (Fig. 1*a*) and by the absence of any polypeptides in other RNA fractions upon SDS/polyacrylamide-gel electrophoresis (Fig. 1*c*). Nevertheless, a polypeptide of M_r 62000 can be recognized before immunoprecipitation as one of the major polypeptides synthesized from fraction 20 mRNA (Fig. 1*b*, indicated by an arrow).

The results from this study indicate that the mRNA coding for mouse PES has a sedimentation coefficient of 17S (Fig. 1*a*) and that a unique polypeptide of M_r 62000 is synthesized on this mRNA (Fig. 1*c*). A 17S mRNA will have approx.

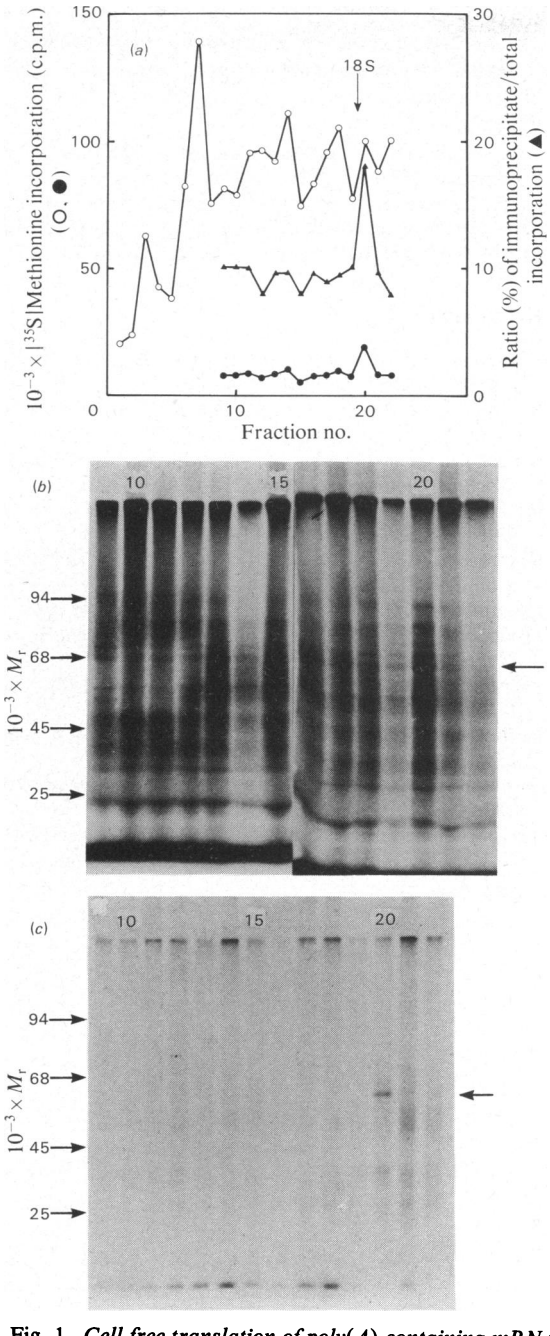

Fig. 1. *Cell-free translation of poly(A)-containing mRNA for PES*

(*a*) Template activity of poly(A)-containing mRNA analysed by sucrose density gradient. The template activity of poly(A)-containing mRNA (1.5–3.0 μg) of each fraction was determined by incorporation of [^{35}S]methionine into protein in a cell-free system from rabbit reticulocytes as described in the Experimental section. ○, Total incorporation into the acid-insoluble fraction after reaction; ●, immunoprecipitation after reaction with antibody against PES; ▲, ratio (%) of the radioactivity of immunoprecipitate to total incorporation. (*b*) Fluorogram of [^{35}S]methionine-labelled polypeptides synthesized in a reticulocyte cell-free system and separated in an SDS/polyacrylamide gel. Total [^{35}S]methionine-labelled translation products synthesized in a reticulocyte cell-free system from the poly(A)-containing mRNA fractions shown in (*a*) were analysed by 8.75% SDS/polyacrylamide-gel electrophoresis. The fluorogram was developed with En^{3}hance (New England Nuclear). The numbers on the fluorogram correspond to the fraction numbers of (*a*). An arrow indicates the presumed subunit of mouse PES, identified in (*c*). (*c*) Immunoprecipitation from cell-free translation products synthesized from poly(A)-containing mRNA. Translation products were precipitated with antibody against PES in the presence of *Staphylococcus aureus* protein A and analysed by SDS/polyacrylamide-gel electrophoresis and fluorography. The numbers on the fluorogram correspond to the fraction numbers of (*a*).

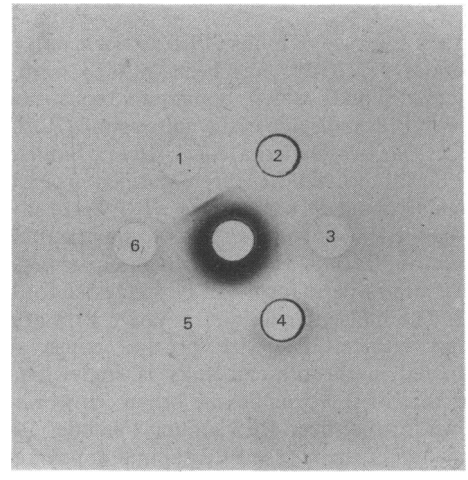

Fig. 2. *Double immunodiffusion test of PES from mouse mastocytoma cells*

Centre well: partially purified antibody from antiserum of rabbit immunized with purified PES from bovine vesicular gland; well 1, purified bovine vesicular gland PES (24 μg of protein); well 3, partially purified (by DEAE-cellulose column chromatography) PES from mouse mastocytoma cells treated with *n*-butyrate (24 μg of protein); well 4, partially purified (by DEAE-cellulose column chromatography) prostaglandin isomerase from mouse mastocytoma cells treated with *n*-butyrate (40 μg of protein); well 6, bovine albumin (24 μg of protein).

2000 nucleotides, and this length seems sufficient for accommodating not only the coding region of the about 1600 nucleotides, but also the 3′-hydroxy poly(A) as well as non-coding sequences.

As in the case of mouse PES, a single polypeptide of about M_r 70000 has been purified from sheep vesicular gland, which apparently constitutes the dimeric PES molecule in the native state (Roth *et al.*, 1980; Van der Ouderaa *et al.*, 1977). Similarly, an M_r 75000 protein has been purified from bovine vesicular gland (Koshihara *et al.*, 1981*a*) in which the native PES molecule seems to have a molecular weight of 300000–350000 (Miyamoto *et al.*, 1976). Thus, a tetrameric form can be suggested for bovine PES. The molecular weight of native PES of mouse is not known. The size of the mouse subunit estimated in the present study is somewhat lower than that of sheep or bovine origin. However, as is known for the sheep PES subunit (Van der Ouderaa *et al.*, 1977), the mouse PES subunit appears to be a glycoprotein, because of its positive periodate/Schiff reaction. Glycosylation of this protein, which would not occur in this cell-free system, should result in the increase of its M_r.

Since the isolated PES of sheep and cow, containing identical subunits, exhibit both cyclooxygenase and hydroperoxidase activities (Miyamoto *et al.*, 1976; Roth *et al.*, 1980; Van der Ouderaa *et al.*, 1977), it appears highly probable that the M_r 62000 polypeptide is a single and unique component of mouse PES, having both enzymic activities. However, since we employed an antigen of heterologous origin for the preparation of antibodies, it might be possible, although unlikely, that other polypeptide(s) involved in the formation of a native PES molecule from mouse was not detected by our system. This problem will be solved when the translation *in vitro* can be coupled with an enzyme assay system.

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