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BIOSYNTHESIS AND PROCESSING OF PLACENTAL ALKALINE PHOSPHATASE

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Polypeptides of 61,500 and 64,500 apparent molecular weights were the precursor and fully processed forms of placental alkaline phosphatase monomer synthesized by choriocarcinoma cells in vivo. [^{3}H] Mannose was incorporated into both polypeptides whereas [^{3}H] glucosamine was incorporated mainly into the 64,500-dalton polypeptide, suggesting processing by the addition of glucosamine moieties. In the absence of microsomal membranes, choriocarcinoma mRNA directed the cell-free synthesis of the preprotein form of alkaline phosphatase monomer of apparent M_r = 60,000. The unglycosylated monomer had an apparent M_r = 58,000. In the presence of microsomal membranes, the 60,000-dalton polypeptide was processed to a polypeptide of apparent M_r = 61,500, co-migrating with the precursor form of alkaline phosphatase monomer.

The alkaline phosphatase (EC 3.1.3.1) isoenzyme from human term placenta is a tissue-specific glycoprotein (1, 2). Placental alkaline phosphatase can be distinguished from that of liver, bone, kidney, and the intestinal isoenzyme by immunological and physiochemical methods (2, 3-5). Placental alkaline phosphatase has attracted interest because it appears not only in normal and malignant trophoblastic cells but in many nontrophoblastic tumors (6-9). It has been postulated that placental forms of isoenzyme from term placenta and trophoblastic and nontrophoblastic tumors are encoded by the same gene, and that malignant transformation derepresses the gene for this embryonic protein (9). Understanding the control of synthesis and processing of alkaline phosphatase may give insights into the transformation process.

Numerous fruitful studies have been performed using human choriocarcinoma cells to elucidate the control of alkaline phosphatase expression (10-12).

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Choriocarcinoma alkaline phosphatase and the enzyme from human term placenta are homo-dimers (4, 13). Both enzymes are composed of the same sized subunits, and have the same pH optimum, and immunological reactivity (13). This paper describes the biosynthesis and processing of alkaline phosphatase in choriocarcinoma cells *in vivo* and in a cell-free protein-synthesizing system *in vitro*.

MATERIALS AND METHODS

Cell Culture

JEG-3 choriocarcinoma cells (14) were grown at 37°C in α-modified minimal essential medium supplemented with streptomycin, penicillin, and 4% fetal bovine serum. Cells in mid-logarithmic growth phase were used in this study.

Immunoprecipitation and Polyacrylamide Gel Electrophoresis

Choriocarcinoma cultures in 25-cm^2 flasks were incubated for various intervals with L-[^{35}S] methionine (100 μCi/ml, 1370 Ci/mmol, Amersham Corp., Arlington Heights, IL), [^{3}H] mannose (100 μCi/ml, 5 Ci/mmol, Amersham), or [^{3}H] glucosamine (100 μCi/ml, 22.5 Ci/mmol, Amersham) as described previously (15).

Cell lysates in phosphate-buffered saline containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 500 μg of trypsin inhibitor were prepared as previously described (15). Anti-placental alkaline phosphatase-precipitable polypeptides in the cell lysates were isolated by the direct immunoprecipitation assay with rabbit antiserum against human term placental alkaline phosphatase. Samples of cell lysates were cleared of nonspecific radioactive components by precipitation with human serum and rabbit anti-human serum as described (15). Two μg of alkaline phosphatase were then added to each sample as carrier. Excess antibody to this enzyme was added and the mixtures were incubated for 30 min at room temperature and overnight at 4°C. Precipitation was approximately 90% complete. The immunoprecipitates were sedimented in a Beckman Microfuge for 15 min, washed as described by Roberts and Roberts (16), heated for 5 min at 95°C, and used for electrophoresis in a 10% polyacrylamide slab gel containing SDS (17). Radioactivity was visualized by fluorography (18). Apparent molecular weights were determined using the following [^{14}C] methionine-labeled protein standards obtained from Amersham: Myosin (200,000), phosphorylase B (92,500), bovine serum albumin (69,000), ovalbumin (46,000), and carbonic anhydrase (30,000).

Cell-free Synthesis of Alkaline Phosphatase

Total RNA was extracted by guanidinium thiocyanate-CsCl method of Chirgwin *et al.* (19). Poly(A)$^{+}$RNA was obtained by subjecting the total RNA to oligo(dT)-cellulose affinity chromatography (20).

Choriocarcinoma poly(A)$^{+}$RNA was translated at 30°C for 60 min in the micrococcal nuclease-treated rabbit reticulocyte lysate system (21), using a kit obtained from Bethesda Research Laboratories, Inc., Gaithersburg, MD. Five μg RNA and 400 μCi of L-[^{35}S] methionine were added to the lysate reaction mixture (150 μl). Dog pancreas microsomal membranes (New England Nuclear, Boston, MA) when used were 0.8 A_{260} unit per reaction mixture.

After translation, some samples of the cell-free system were treated with 8 μg each of trypsin and chymotrypsin at 4°C for 60 min. the reaction was terminated by the addition of 500 μg of trypsin inhibitor and 5 mM L-1-tosylamide-2-phenylethyl chloromethyl ketone. Anti-alkaline phosphatase-precipitable polypeptides in the cell-free translation products were isolated by immunoprecipitation.

RESULTS AND DISCUSSION

Pulse labeling human choriocarcinoma cells with L-[^{35}S] methionine demonstrated that a polypeptide of apparent M_r = 61,500 was the first anti-placental alkaline phosphatase-precipitable product synthesized (Fig. 1). An additional anti-alkaline phosphatase-pecipitable polypeptide of apparent M_r = 64,500 appeared after pulses of 60 min or longer. The 64,500-dalton polypeptide co-migrated with the alkaline phosphatase monomer isolated from human term placenta (see Fig. 3, below), and was the major anti-alkaline phosphatase-precipitable product after longer pulses. The radioactivity in

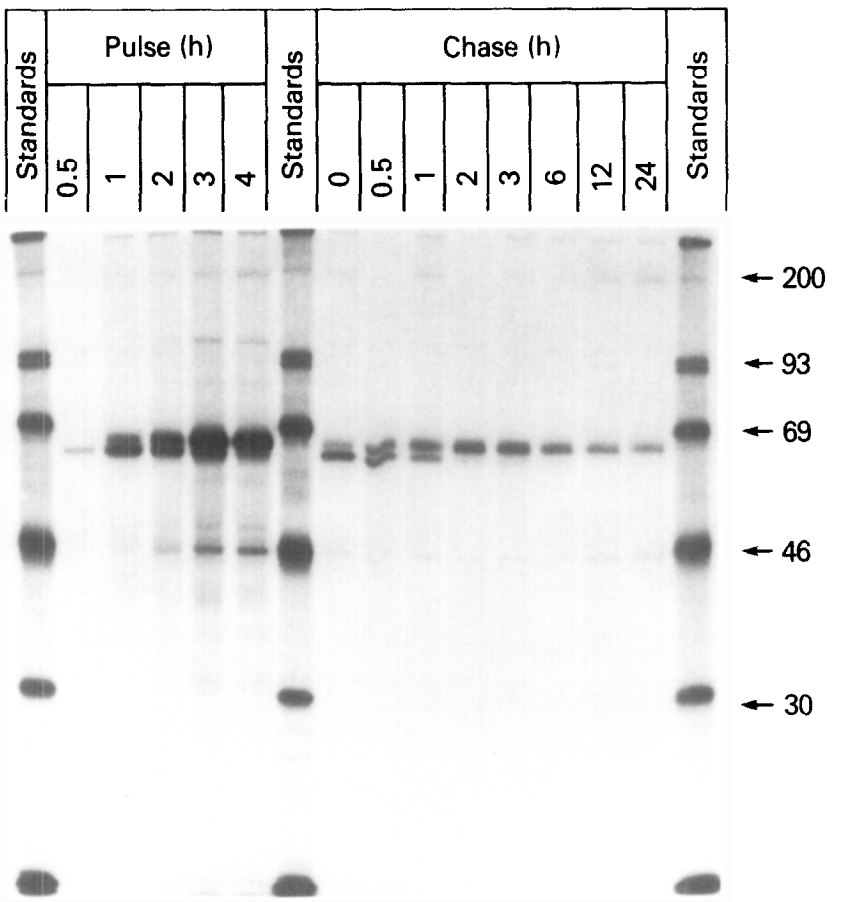

Figure 1. Pulse and pulse-chase labeling of placental alkaline phosphatase. Pulse experiment: cells were labeled with L-[^{35}S] methionine for 0.5, 1, 2, 3, and 4 h. Chase experiment: cells were labeled with L-[^{35}S] methionine for 1 h then subjected to chase with fresh medium for 0.5, 1, 2, 3, 6, 12, and 24 h. Cell lysates were immunoprecipitated with rabbit anti-alkaline phosphatase serum. Anti-alkaline phosphatase-precipitable polypeptides were analyzed by SDS-gel electrophoresis and fluorography.

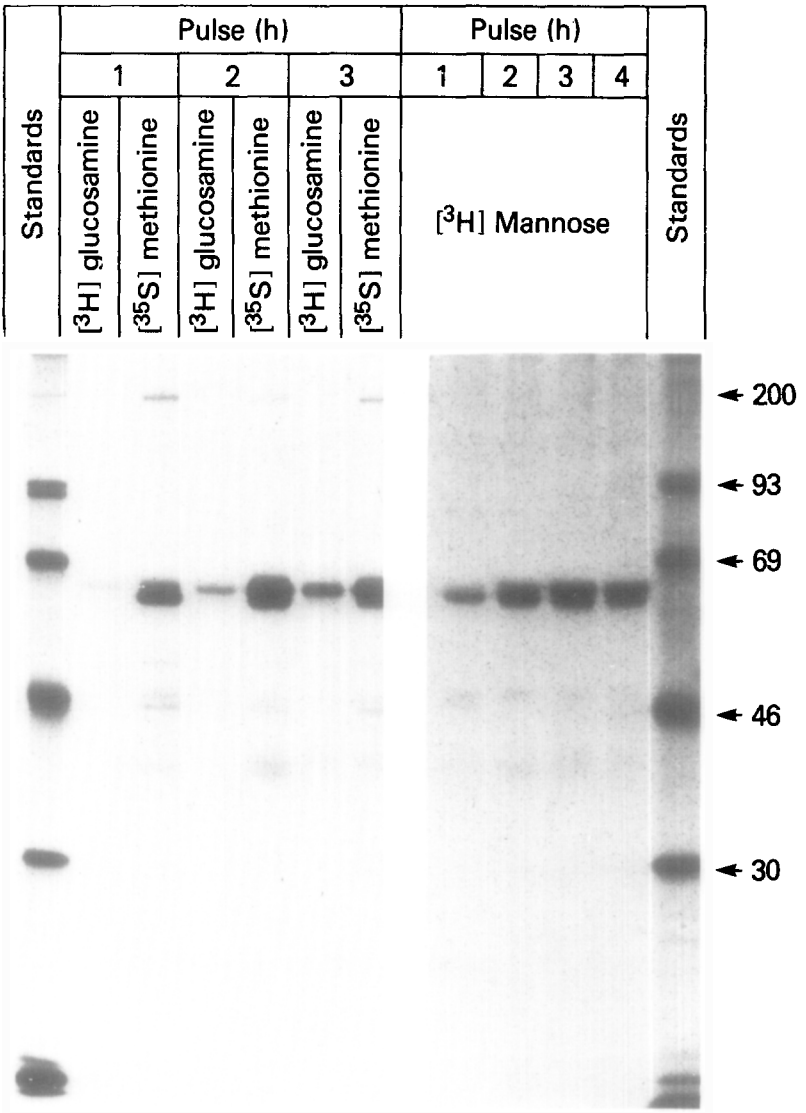

Figure 2. Incorporation of L-[^{35}S] methionine, [^{3}H] glucosamine, and [^{3}H] mannose into anti-placental alkaline phosphatase-precipitable polypeptides. Cells were labeled with L-[^{35}S] methionine or [^{3}H] glucosamine for 1, 2, and 3 h or were labeled with [^{3}H] mannose for 1, 2, 3, and 4 h. Cell lysates were immunoprecipitated with rabbit anti-alkaline phosphatase serum. Anti-alkaline phosphatase-precipitable polypeptides were analyzed by SDS-gel electrophoresis and fluorography.

the 61,500-dalton polypeptide could completely be chased into the 64,500-dalton polypeptide, indicating a precursor-product relationship (Fig. 1).

Choriocarcinoma cells secreted no alkaline phosphatase; no anti-alkaline phosphatase-precipitable radioactivity was found in medium. The specificity of the anti-alkaline phosphatase-precipitable products was demonstrated by

competition for immunoprecipitation by authentic alkaline phosphase from human term placenta. The addition of excess placental alkaline phosphatase prevented immunoprecipitation of both the 61,500- and 64,500-dalton polypeptides (data not shown).

Both [^{3}H] mannose and [^{3}H] glucosamine were incorporated into the anti-alkaline phosphatase-precipitable products, suggesting that the choriocarcinoma phosphatase is a glycoprotein, as is the alkaline phosphatase from term placenta. The kinetics of [^{3}H] mannose incorporation into anti-alkaline phosphatase-precipitable polypeptides were similar to the kinetics of [^{35}S] methionine incorporation (Fig. 2). The [^{3}H] mannose-labeled polypeptides of 61,500 and 64,500 apparent molecular weights also appeared in sequence. However, [^{3}H] glucosamine appeared to be incorporated mainly into the 64,500-dalton polypeptide (Fig. 2). It appears that the conversion of 61,500- to 64,500-dalton polypeptides involved the incorporation of additional glucosamine moieties.

It has been shown that many secretory and membrane proteins are synthesized initially as larger molecular weight precursors (preproteins) that contain an amino-terminal extension termed a signal sequence (22, 23). The preproteins are segregated and processed within the microsomal vesicles that results in the cleavage of the signal peptide and core-glycosylation (24-27). This was demonstrated largely by using cell-free protein-synthesizing systems, because of the rapid cleavage to the final product by cells *in vivo*.

Accordingly, we examined cell-free translation of alkaline phosphatase directed by choriocarcinoma poly(A)$^+$RNA (Fig. 3). In the absence of microsomal membranes, a polypeptide of apparent M_r = 60,000 was the translation product immunoprecipitated with anti-alkaline phosphatase serum (Fig. 3, lane 6). This polypeptide was 2,000-dalton greater in apparent molecular weight than the unglycosylated anti-alkaline phosphatase-precipitable polypeptide of apparent M_r = 58,000 (Fig. 3, lane 5). The latter was synthesized by choriocarcinoma cells *in vivo* in the presence of the

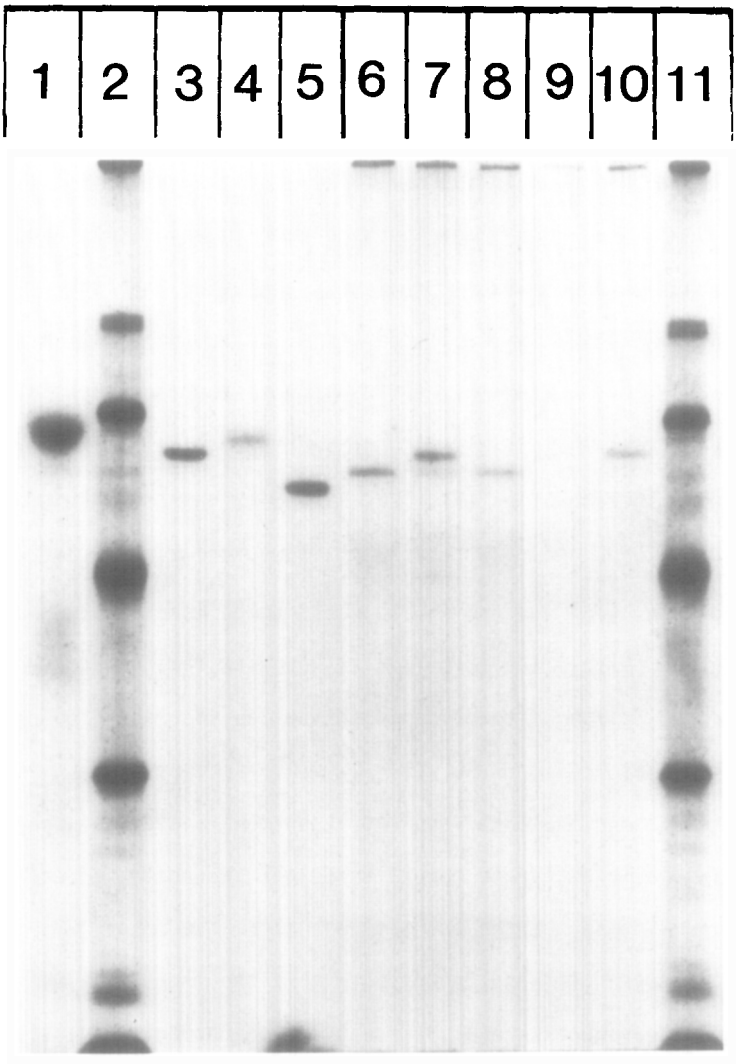

Figure 3. Biosynthesis and processing of placental alkaline phosphatase in a cell-free system. *In vitro* translation of choriocarcinoma poly(A)$^+$RNA in a rabbit reticulocyte lysate system was described under Materials and Methods. Anti-placental alkaline phosphatase-precipitable polypeptides in the translation products were analyzed by SDS-gel electrophoresis and fluorography. Lane 1, [^{125}I]-labeled placental alkaline phosphatase monomer; lane 2 and 11, [^{14}C] methionine labeled molecular weight standards; lanes 3 and 4, precursor (lane 3) and fully processed (lane 4) anti-alkaline phosphatase-precipitable polypeptides synthesized by choriocarcinoma cells *in vivo*; lane 5, unglycosylated anti-alkaline phosphatase-precipitable polypeptide synthesized by choriocarcinoma cells in the presence of tunicamycin; lane 6 to 10, anti-alkaline phosphatase-precipitable polypeptides from *in vitro* translation products: microsomal membranes were absent during translation (lane 6), microsomal membranes were present during translation (lane 7), microsomal membranes were added after translation (lane 8), trypsin and chymotrypsin were added after translation in the absence of microsomal membranes (lane 9), and trypsin and chymotrypsin were added after translation in the presence of microsomal membranes (lane 10).

glycosylation inhibitor tunicamycin (28). These data suggest that alkaline phosphatase monomer was also synthesized initially as a preprotein.

In the presence of dog pancreas microsomal membranes, the 60,000-dalton polypeptide was processed to a anti-alkaline phosphatase-precipitable polypeptide of apparent M_r = 61,500 which co-migrated with the precursor form of alkaline phosphatase monomer synthesized by choriocarcinoma cells in vivo (Fig. 3, compare lane 7 and 3). The addition of excess placental alkaline phosphatase also prevented immunoprecipitation of the 60,000- and 61,500-dalton polypeptides synthesized in vitro (data not shown). Our data strongly suggest that the cell-free system was reproducing those features of the cell necessary for the correct synthesis of alkaline phosphatase. Since the 61,500-dalton polypeptide was a glycoprotein containing mannose residues (see Fig. 2), core-glycosylation occurred in the presence of microsomal membranes. Further, such processing was occurring cotranslationally; the 60,000-dalton polypeptide was the only anti-alkaline phosphatase-precipitable product observed if microsomal membranes were added after translation (Fig. 3, lane 8).

The 60,000-dalton polypeptide synthesized in the absence of microsomal membranes was sensitive to added protease (Fig. 3, lane 9). However, the 61,500-dalton polypeptide synthesized in the presence of microsomal membranes was resistant to digestion (Fig. 3, lane 10). Therefore, the preprotein form of alkaline phosphatase monomer was transferred and processed in the microsomal vesicles as are many other secretory and membrane proteins (24-27, 29, 30).

The results of studies in vitro and in vivo showed that placental alkaline phosphatase was first assembled in the membrane of the endoplasmic reticulum. Pulse chase experiments have established that the microsomal form of alkaline phosphatase was the precursor of the higher molecular weight fully processed phosphatase found in the plasma membrane.

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