Expression of different-sized placental alkaline phosphatase mRNAs in placenta and choriocarcinoma cells

cDNA clone/mRNA splicing/protein compartmentation)

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ABSTRACT The expression of human placental-type alkaline phosphatase (ALPase) in the placenta and in three choriocarcinoma cell lines was examined by translation in vitro and RNA blot analysis using a cDNA for placental ALPase. Placental RNA directed the synthesis of two polypeptides that could be immunoprecipitated with antisera to placental ALPase. Translation of RNA from the choriocarcinoma cell lines, with or without sodium butyrate treatment, yielded a single immunoprecipitable product of molecular weight intermediate between those of the products from the placenta mRNA. Two cDNA clones for placental ALPase were isolated by antibody screening of a placental cDNA library constructed in λgt11. The overlapping cDNAs include 462 nucleotides of coding sequence. RNA blot analysis has confirmed that induction of placental-type ALPase levels during placental development is accompanied by an increase in steady-state placental ALPase mRNA concentrations. Examination of the mRNAs revealed a placental ALPase mRNA of 3.0 kilobases (kb) and a distinct choriocarcinoma placental-type ALPase mRNA of 2.6 kb, implying that transformation of normal to malignant trophoblast is associated with the expression of a distinct placental-type ALPase gene transcript and its protein product.

Human placental alkaline phosphatase (ALPase) is one of at least three tissue-specific isoenzymes, which are distinguished by immunological, electrophoretic, and biochemical criteria (reviewed in ref. 1). The active form of the placental enzyme is a dimer composed of identical subunits of Mr ≈ 65,000 (1). Placental ALPase is one of the predominant glycoproteins located at the membrane surface of placental microvilli (2) and, in addition, is present at high concentrations in the maternal serum during pregnancy (3). These observations suggest that placental ALPase is both a membrane-bound and a secreted protein.

Earlier studies have indicated that ALPase expression is developmentally regulated in the placenta. The ALPase present in the placenta before the 10th week of gestation resembles the adult human liver isoenzyme (4, 5). At 10-13 weeks, the placental isoenzyme becomes detectable, and by 14 weeks, accounts for all the ALPase activity in the placenta (4). Thus, a developmental switch occurs, in which early expression of liver-type ALPase is followed by the induction and subsequent increase of placental-type ALPase levels throughout pregnancy (4).

Placental ALPase was one of the first proteins found to be ectopically expressed by cancer cells (6) and has been observed in tumors of trophoblastic and nontrophoblastic origin (1, 7). Although expression of placental ALPase in tumors has been attributed to derepression of an embryonic gene (1), it has not been established that the placental-type enzymes synthesized in tumor cells and normal placenta are products of the same gene. Monoclonal antibody crossreactivity (8) and sequence analysis of the NH2 terminus (9) indicate that there is structural identity between the placental enzyme and the enzyme produced in some nontrophoblastic tumors. However, many tumors, including choriocarcinomas, which are malignant trophoblastic cells, synthesize a placental ALPase-like enzyme that differs from the placental enzyme in various biochemical properties (10-12). It has been proposed that a distinct gene encoding a placental ALPase-like enzyme may be expressed in these tumors (13).

The level of placental-type ALPase expression in choriocarcinomas is much lower than in the placenta (3, 7). Treatment with sodium butyrate causes a significant induction of placental ALPase-like enzyme activity in choriocarcinoma cells (14). The mechanism of this induction is unclear but is presumed to be at the level of mRNA transcription (14). Sodium butyrate can therefore be used to increase placental ALPase expression in these cells to easily detectable levels.

Initially, we observed that placental and choriocarcinoma RNAs direct the synthesis of three distinct primary translation products that were precipitable with antisera to placental ALPase. To examine their differences in more detail, we isolated two cDNA clones for placental ALPase from term placental RNA. Using the cDNAs as probes, we found that the induction of placental ALPase levels during placental development is due to an increase in steady-state mRNA concentration. In addition, we have found that choriocarcinoma cells express a placental ALPase mRNA that is apparently ~ 0.4 kilobase (kb) shorter than the mRNA of term placenta.

MATERIALS AND METHODS

Materials. Placental ALPase was purchased from Sigma; reverse transcriptase, from Life Sciences; restriction endonucleases, DNA ligase, and DNA polymerase, from New England Biolabs or Bethesda Research Laboratories; EcoRI-digested Agt11 arms, from Promega Biotec (Madison, WI); a packaging extract kit, from Amersham; and EcoRI octanucleotide linkers, from P-L Biochemicals.

Cell Culture. JEG-3, JAr, and BeWo choriocarcinoma cell lines were grown as monolayers in minimal essential medium supplemented with 10% fetal bovine serum. Confluent monolayers were incubated in the presence of 5 mM sodium butyrate for 16-18 hr and harvested for RNA isolation (15).

RNA Isolation. RNA from first trimester (8-12 wk) and term placentas was isolated by a modification of the guaniinium/cesium chloride method (16). Poly(A+) RNA was

Abbreviations: ALPase, alkaline phosphatase; kb, kilobase(s).
prepared by oligo(dT)-cellulose chromatography (16). RNA was isolated from cultured cells as described (15).

Protein Sequence Determinations. Commercial placental ALPase was further purified by methods previously described (17). The protein was subjected to reduction and carboxymethylation, followed by trypsin digestion (18). The tryptic peptides were separated by HPLC (18). After lyophilization, the purified peptides were subjected to automated Edman degradation in an Applied Biosystems gas-phase sequencer using the "no vac" program supplied by the manufacturer. Separation and quantification of the phenylthiohydantoin amino acid derivatives was by HPLC (18).

Cell-Free Translation. Total RNA (10 μg) was translated in either an ascites tumor-cell (19) or reticulocyte (20) lysate containing [35S]methionine (1200 Ci/mmol; 1 Ci = 37 GBq). For immunoprecipitation, the translation reaction mixture was treated with rabbit antiserum to placental ALPase followed by Staphylococcus aureus protein A as described (21). Characterization of the antiserum has established that it does not crossreact with liver ALPase (5). Translation of mRNA in the presence of microsomal membranes was according to published methods (19).

cDNA Library Preparation and Screening. Double-stranded cDNA was prepared from term placental poly(A)+ RNA by the method of Gubler and Hoffman (22). EcoRI octanucleotide linkers were added to the cDNA inserts, which were then ligated into EcoRI-digested Agt11 arms and packaged in vitro. The Agt11 expression library was screened with antiserum to placental ALPase (23).

Nucleic Acid Sequencing and RNA and DNA Blot Analysis. Nucleic acid sequencing was performed by the dideoxy chain-termination method (24) after subcloning in M13 vectors. Either the universal M13 sequencing primer or synthetic oligonucleotides (25) derived from the placental ALPase cDNA were used for sequence determination of both strands. Cloned DNA inserts used for hybridizations were isolated from the phage vector and labeled to high specific activity by the method of Feinberg and Vogelstein (26). RNA blots were prepared for hybridization analysis as described (15). Densitometric scanning of the autoradiographs was performed with a microdensitometer (Joyce-Loebl). Human genomic DNA was digested with restriction enzymes, blotted onto nitrocellulose, and hybridized as described (16).

RESULTS

To examine the regulation of placental-type ALPase expression in the placenta during gestation, we analyzed the levels of placental-type ALPase mRNA at first trimester and term by in vitro translation. Total RNA was translated in either reticulocyte or ascites tumor-cell lysate. The [35S]methionine-labeled products were precipitated with placental ALPase-specific antiserum and analyzed by gel electrophoresis. Immunoprecipitation of translation products from term placental RNA yielded two distinct bands at Mr 61,000 and 63,500 (Fig. 1, lane 3). Additional bands in this lane were also present after precipitation with normal rabbit serum (lanes 4 and 5). The addition of purified, unlabeled placental ALPase to the reaction mixtures resulted in decreased immunoprecipitation of both labeled placental ALPase species, whereas the nonspecific bands remained unaffected (lanes 5 and 6). Quantitation of radioactivity obtained by elution of the two bands indicates that placental ALPase mRNA in term placenta is ~0.05% of the total mRNA.

To investigate whether the two bands result from co- or posttranslational modification of a single product of placental ALPase mRNA, we added ascites microsomal membranes to the translation reactions. The membranes are capable of signal-peptide removal and asparagine-linked core glycosylation (27). We observed an increase of ~2000 in the apparent molecular weight of both ALPase polypeptides (Fig. 2), consistent with core glycosylation of both translation products. Because glycosylation requires cotranslational translocation (27), this result suggests that both modified proteins are sequestered within the membrane vesicles. It is unlikely that one is derived from the other by proteolysis or by other processing event. Translation in vitro for 15 min to 6 hr consistently yielded both proteins (data not shown). Moreover, both proteins have been observed after translation of RNA from over 30 individual placenta and are usually present in equal amounts. These results strongly suggest that the two proteins are translated from separate mRNAs, both encoding placental-type ALPase.

In contrast to term placental RNA, no detectable placental ALPase product was present in the translation products of first-trimester RNA (Fig. 1, lanes 2 and 3). This result confirms that there is an increase in translatable placental
ALPase mRNA during gestation, consistent with earlier findings for placental ALPase levels (4).

The placental ALPase translation products from term placental mRNA were compared to those of choriocarcinoma mRNA. RNA was isolated from the JEG, JAr, and BeWo choriocarcinoma cell lines before and after treatment with 5 mM sodium butyrate. mRNA from all three cell lines encodes an immunoprecipitable polypeptide of Mr ~ 62,000, which is intermediate in size between the two translation products from term placental RNA (Fig. 3, lanes 2–5). The significance of the slight difference in mobility of the JAr and BeWo proteins (lanes 3 and 4) is unclear. RNA from control BeWo cells encodes a polypeptide of the same molecular weight as that from sodium butyrate-treated BeWo cells (lanes 4 and 5), excluding the possibility that sodium butyrate treatment is responsible for the distinct choriocarcinoma translation product. Sodium butyrate increases the amount of the immunoprecipitable translation product about 10-fold in BeWo cells (lanes 4 and 5). Addition of microsomal membranes resulted in an upward shift of the single placental ALPase band, indicating that it undergoes core glycosylation (data not shown), which is consistent with a previous report (28). From liquid scintillation counting of immunoprecipitable radioactivity, we estimate that placental ALPase mRNA comprises about 0.5% of the total mRNA in sodium butyrate-treated BeWo cells. Thus, choriocarcinoma mRNA encodes a form of placental ALPase that is immunologically related to the two placental ALPase species encoded by RNA from term placenta but that is distinct from both, as shown by the difference in apparent molecular weight of the primary translation products.

To investigate the relationship between the placental ALPase translation products from term placenta and choriocarcinoma cells, we isolated cDNAs to term placental mRNA. A cDNA library was generated from human term placental RNA and inserted into the λgt11 expression vector. The library was screened with the placental ALPase antisera. The cDNA inserts of two positive phage clones were placed into M13 vectors and their nucleotide sequences were determined on both strands (Fig. 4). The two cDNA inserts of 370 and 600 base pairs contain an overlapping region of 180 base pairs. To confirm the identity of the cDNA clones, we compared their predicted amino acid sequences to sequences determined from tryptic fragments of placental ALPase. Purified placental ALPase was subjected to trypsin digestion and, following separation by HPLC, the amino acid sequences of 12 individual tryptic fragments, comprising 110 residues, were determined (data not shown). Examination of the predicted amino acid sequences of the putative cDNA clones revealed an open reading frame of 462 bases, which ends with a termination codon. Predicted amino acid sequences corresponding precisely to those of two of the tryptic peptides were found within this reading frame (underlined in Fig. 4), thus indicating that the partial cDNA clones encode 154 amino acids corresponding to the COOH-terminus of placental ALPase.

The cDNAs were used as probes for analysis of placental and choriocarcinoma mRNAs. Both the cDNAs hybridize to a single mRNA band of 3.0 kb on term placental RNA blots (Fig. 5, lanes 2 and 4). The detection of only one band does not support our suggestion, based on translation data, that there are two placental ALPase mRNAs in the placenta. However, if the mRNAs were similar in size, they might not be resolved by agarose gel electrophoresis. Alternatively, it is possible that our partial cDNAs recognize only one of the two mRNAs. To address this question, we generated an unambiguous 39-mer synthetic oligonucleotide encoding the NH2-terminal amino acid sequence of placental ALPase (29), based on known codon-usage frequencies. Because there is no reported heterogeneity in the NH2-terminal sequence of placental ALPase, we expected that both mRNAs should be recognized by this probe. The labeled oligonucleotide was hybridized to blots of both total and poly(A)+ term placental RNA. The mRNA band detected with this probe was identical in size to that recognized by the cDNA probe (data not shown). We conclude that if there are two separate mRNAs, they must be very similar in size and will only be distinguished by isolation and sequence analysis of corresponding cDNAs.

Placental ALPase mRNA was undetectable in first-trimester total RNA (Fig. 5, lane 3). More sensitive assays with poly(A)+ RNA (lane 1) and RNA dot blot hybridizations (data not shown) revealed a low level of placental ALPase mRNA at this stage. Comparison of the first-trimester and term mRNAs (lanes 1–4) showed that the developmental expression of ALPase in the placenta is regulated through steady-state mRNA levels. We observed a 7- to 10-fold induction in placental ALPase mRNA levels between first-trimester and term placentas, as measured by scanning densitometry of the autoradiographs.

To examine the placental ALPase mRNA from choriocarcinoma cells, a term placental ALPase cDNA was used to probe blots of RNA isolated from control and sodium butyrate-treated cultures. The cDNA hybridized to mRNA from both of the choriocarcinoma cell lines examined (Fig. 5, lanes 5–7). However, comparison of the placental ALPase mRNAs showed that the length of the choriocarcinoma mRNA is 2.6 kb, whereas that of the placental mRNA is 3.0 kb. The tumor-specific mRNA was also detected when the oligonucleotide corresponding to the NH2-terminal sequence was used as a probe (data not shown), indicating homology between the NH2-terminal sequences of the choriocarcinoma and term placental ALPases. Prolonged exposure of the RNA blots revealed an mRNA of identical size in uninduced BeWo cells (data not shown), ruling out the possibility that sodium butyrate treatment is responsible for the smaller mRNA in choriocarcinoma cells. These results also showed that the induction of placental ALPase enzyme levels by sodium butyrate is due to an increase in steady-state placental ALPase mRNA concentrations (Fig. 5, lanes 6 and 7).

Examination of the number and structure of the genes encoding placental-type ALPase is necessary to determine the relationship between the placental ALPase mRNAs from term placenta and choriocarcinoma. The placental ALPase cDNAs were used for Southern blot analysis of human genomic DNA digested with two restriction enzymes (Fig. 6). Because one to three hybridizing bands were observed, we suggest that there are at most three genes encoding placental-type ALPase.
FIG. 4. Nucleotide sequence of the cDNA clones. The sequences of the two clones overlap between nucleotides 170 and 370. The predicted amino acid sequence (shown below the nucleotide sequence, in the one-letter amino acid code) shows an open reading frame extending for 462 bases, terminated by a stop codon (asterisk). Predicted amino acid sequences confirmed by automated Edman degradation of placental ALPase tryptic peptides are underlined. The cDNAs thus encode 154 amino acids of the COOH-terminus of placental ALPase.

DISCUSSION

Translation of term placental RNA yields two immunoprecipitable placental-type ALPase products, of Mr 61,500 and 63,000. A similar result has been obtained with rat intestinal mRNA, which directs the synthesis of two translation products specific for rat intestinal ALPase (30). This is significant because the intestinal ALPase, like the placental-type enzyme, is localized to microvillar membranes and is also secreted (1). Based on our observations, we suggest that the two placental ALPase polypeptides represent the primary translation products of separate mRNAs. Because we detect only a single placental ALPase mRNA band on blots of term placental RNA, we conclude that the two mRNAs are nearly identical in size.

It has been found that placental-type ALPase isolated exclusively from microvillar membranes differs from total placental ALPase in its apparent molecular weight and degree of hydrophobicity (31). However, no heterogeneity has been detected within the known amino acid sequence of placental ALPase (29). These observations suggest that there are two closely related forms of placental-type ALPase in the placentae—the form that is localized to the microvillar membrane surface (31) and a lower molecular weight form that may correspond to secreted placental ALPase. Because of their immunologic crossreactivity and similar molecular weights, the two translation products from term placental RNA may represent the membrane and secreted forms of the enzyme. The slight difference in apparent molecular weight between the primary translation products probably indicates a difference in the length of mRNA coding sequence. The membrane form of the enzyme could be encoded by an mRNA identical to that of the secreted form, except for an extra membrane-spanning region. Differential splicing of one gene transcript could generate two closely related mRNAs, by replacement of a single exon, as occurs in the synthesis of the secreted and membrane-bound forms of immunoglobulin heavy chain (32). Alternatively, the two placental ALPase mRNAs may be transcribed from separate genes. Our preliminary results indicate that the placental ALPase gene copy number is low, but more definitive quantitation is required to distinguish between these possibilities.

Both translation data and RNA blot analysis demonstrate that placental ALPase mRNA levels are developmentally regulated in the placenta, consistent with previous reports showing induction of placental ALPase enzyme levels during placental maturation (4, 5). Quantitative analysis using densitometric scanning shows a 7- to 10-fold increase in steady-state placental ALPase mRNA levels between first trimester and term. This increase may be due to changes in the rate of transcription or in the stability of placental ALPase mRNA. In addition, induction of placental ALPase during gestation may be related to the state of trophoblast differentiation. Immunofluorescent-antibody studies suggest that the enzyme is synthesized in the syncytiotrophoblast (33). Beginning in the first trimester, mononucleated cytrophoblasts fuse to form the syncytiotrophoblast, which is the dominant trophoblast.
component at term (34). Placental ALPase mRNA concentrations may directly reflect the mass of syncytiotrophoblast in the placenta at gestation proceeds.

The placental-type ALPase present in choriocarcinoma cells behaves as a membrane-bound protein (12), and these cultured cells do not secrete the enzyme (35). Taken together, these observations suggest that, unlike the placenta, choriocarcinoma cells synthesize only one form of placental-type ALPase, a conclusion supported by our translation data. We find that the single translation product of choriocarcinoma placental-type ALPase mRNA is distinct from both of the placental ALPase products. The intermediate-size translation product of choriocarcinoma cells probably reflects a difference in the length of the mRNA coding sequence, which may account for the altered biochemical properties of ALPase reported for these cells (11, 12). We observed a placental-type ALPase mRNA species that is specific to choriocarcinoma cells. This mRNA is estimated to be 2.6 kb long, whereas the placental ALPase mRNA is 3.0 kb. The length of the mRNA required to code for any of the placental-type ALPase translation products is only about 1.6 kb. This suggests that the placental-type ALPase mRNAs contain an untranslated sequence up to 1.4-kb long, and alterations in the length of this noncoding sequence may contribute to the significant size difference between the placental and choriocarcinoma mRNAs.

The aberrant expression of developmentally regulated proteins, such as placental ALPase, by neoplastic cells is thought to result from derepression of the normal gene (1). However, the synthesis of a distinct placental-type ALPase mRNA in choriocarcinoma cells suggests that placental-type ALPase expression in these cells is due to events other than derepression of the gene encoding placental ALPase. However, because of the trophoblastic origin of choriocarcinoma, we cannot exclude the possibility that the choriocarcinoma mRNA represents a form of the enzyme that is transiently expressed during normal placental differentiation.

The choriocarcinoma mRNA may be derived from the normal placental transcript by a tumor-specific splicing pattern. Alternatively, a separate gene might account for the variant placental ALPase-like enzymes found in many tumors (13), and the distinct mRNA we detected in choriocarcinoma cells may provide direct evidence for this gene. Further examination is required to distinguish between these possibilities. In either case, we have shown that transformation of normal trophoblast to malignant tumor cell is associated with the expression of a distinct placental-type ALPase gene transcript and its protein product.

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