Expression of a Nagao-type, Phosphatidylinositol-Glycan Anchored Alkaline Phosphatase in Human Choriocarcinomas

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ABSTRACT

The alkaline phosphatase (AP) synthesized by human tumor cells closely resembles human placental AP (PLAP). Little is known about the molecular events that lead to the expression of a placenta-like AP in tumor cells. The complementary DNA encoding the AP expressed by a choriocarcinoma cell line, BeWo, was isolated and characterized. The complementary DNA is the product of the germ cell AP (Nagao isozyme) gene and not of the term PLAP gene. Like placental AP, the tumor AP can be released from the cell membrane by a phosphatidylinositol-specific phospholipase C and has a phosphatidylinositol-glycan (PI-glycan) moiety at the COOH terminus. Immunoprecipitation of phosphatidylinositol-specific phospholipase C-treated AP and analysis by polyacrylamide gel electrophoresis or isoelectric focusing demonstrates that at least 95% of the AP contains PI-glycan. Two-dimensional gel electrophoresis reveals two precursors of the mature AP. One of these does not bind an antibody against the Trypanosoma variable surface glycoprotein cross-reacting determinant and probably does not contain PI-glycan. This precursor had a shorter half-life than the more prominent PI-glycan-containing precursor in pulse-chase experiments, suggesting a precursor-product relationship between the two proteins. These data demonstrate that BeWo AP is the product of a gene normally expressed in testis, thymus, and germ cells, but not in placenta. Thus, the expression of BeWo AP results from the repression of the PLAP gene and derepression of the germ cell AP gene and, as such, the expression is ectopic. The BeWo AP (Nagao isozyme) is modified with PI-glycan that is added soon after translation, not cotranslationally.

INTRODUCTION

Human PLAP2 is frequently expressed in tumor cells of both trophoblastic and nontrophoblastic origin (1). The synthesis of PLAP in a nontrophoblastic tumor was first observed in a squamous cell carcinoma of the lung (2). The PLAP (Regan isozyme) produced by this tumor was identical to the normal, term PLAP by immunological, electrophoretic, and enzymatic criteria. The evidence suggested that the tumor PLAP was the product of the same gene encoding normal PLAP and that the expression of the Regan isozyme by these tumor cells resulted from the derepression of the normal PLAP gene rather than the expression of a separate tumor-specific gene.

Subsequently, a distinct placenta-like AP (Nagao isozyme) was described in a variety of other tumors (3). The enzyme shared some biochemical and antigenic properties with PLAP but differed in possessing greater sensitivity to heat inactivation, inhibition by EDTA and l-leucine, and unique antigenic sites (1). The enzyme is expressed in small amounts by testis (4), germ cells (5), and thymus (6) and is highly expressed by germ cell tumors such as seminomas. The gene encoding the Nagao isozyme was recently cloned from a seminoma library and is distinct from the normal, term PLAP gene (7). The authors suggest that the AP produced by seminomas results from the enhanced expression of a gene normally expressed at low levels in germ cells rather than from the reexpression of a gene that is not expressed in the cell lineage from which these tumors arise, as postulated for the Regan isozyme.

Choriocarcinomas also express a placenta-like AP that is indistinguishable from the Nagao isozyme by biochemical and immunological criteria, suggesting that choriocarcinomas express the germ cell AP gene rather than the PLAP gene (8, 9). The possibility that the choriocarcinoma AP may differ from the Nagao isozyme was raised by the observation that patients with seminomas have high levels of the Nagao isozyme in their serum, whereas patients with choriocarcinomas generally do not have elevated serum AP (10–12). One explanation of this finding is that the seminoma AP is secreted, whereas the choriocarcinoma AP is membrane bound. APs from various tissues, including placenta, are anchored in the cell membrane by a phosphatidylinositol-glycan moiety (13). The group is covalently bound to the COOH terminal amino acid of the mature protein. Comparison of protein sequence data for PLAP and other PI-glycan-linked proteins suggests that the PI-glycan is added after the removal of 20–30 amino acids from the COOH terminus. Interestingly, the Nagao isozyme contains two amino acid changes in the COOH terminal sequence that is cleaved during addition of PI-glycan. Both differences involve the substitution of uncharged amino acids for charged amino acids. Because the recognition site for the enzyme(s) that processes the COOH terminal has not been identified, a prediction about the effects of the amino acid changes on PI-glycan addition cannot be made. It is possible that the Nagao isozyme as encoded in germ cells does not contain PI-glycan, resulting in secretion of the AP.

A cultured choriocarcinoma cell line, BeWo, expresses a butyrate-inducible AP that is biochemically and immunologically identical to the Nagao isozyme (14, 15). The AP protein and mRNA in the butyrate-induced cells are indistinguishable from that in the uninduced cells (15, 16). The mRNA expressed by these cells is detectable by probes derived from PLAP but is about 400 base pairs smaller than the mRNA present in term placenta (16). To determine whether these choriocarcinoma cells have enhanced expression of normal term PLAP or expression of the Nagao isozyme gene as a result of malignancy, we have isolated the cDNA encoding the choriocarcinoma AP of BeWo cells and have characterized the membrane anchor of the BeWo AP. The cDNA is identical to that predicted from the BeWo gene. Both the BeWo AP and the AP encoded by the cDNA are posttranslationally modified by PI-glycan.

MATERIALS AND METHODS

cDNA Library Construction and Clone Isolation. Total RNA was isolated from BeWo cells by the SDS-proteinase K method (17). Poly-
adenylate-containing RNA was prepared on an oligo d(T) column (18). Double-stranded cDNA was synthesized from 10 \( \mu \)g of mRNA and ligated into EcoRI cut \( \lambda g t 11 \) arms (19). Plaques were screened with a random primer-labeled probe derived from the 5'-end of a human PLAP cDNA previously isolated in this laboratory (data not shown). Positive clones were subcloned into \( \Phi g E M \) and sequenced fully by the dideoxynucleotide method (20).

Cell Culture. BeWo cells (American Type Culture Collection CCL 98) were grown in Earle's MEM supplemented with 10% fetal bovine serum (Cell Culture Laboratories), glutamine, penicillin, and streptomycin. Twenty-four h prior to an experiment, confluent cultures were changed to medium containing 2 mm sodium butyrate and 10 mm Hepes (pH 7.0) to induce alkaline phosphatase synthesis (16). The butyrate-induced cells have slower growth than the uninduced cells. A generalized increase in protein and mRNA levels is not seen and some protein levels are decreased; for instance, the \( \alpha \)-subunit of human chorionic gonadotropin is decreased (15).

Cell Transfection. Full-length insert was subcloned into the eukaryote expression vector, \( \Phi S V L \). Five \( \mu \)g was transfected into COS-1 cells on 100-mm dishes with DEAE-dextran according to a published procedure (21). The cells were harvested 72 h after transfection.

Metabolic Labeling. For protein labeling, confluent cells in T-75 flasks were incubated at 37°C with methionine-free Earle's MEM containing 2 mm sodium butyrate for 30 min. The medium was replaced with 5 ml of identical medium containing 50 \( \mu \)Ci/ml of \( ^{[35S]} \)methionine (1000 Ci/mmol), and the cultures were incubated for the indicated times. Chases were done with Earle’s MEM containing a 100-fold excess of unlabeled methionine.

Immunoprecipitation of Alkaline Phosphatase. Cell lysates were prepared by washing the cell layer 3 times with PBS and then scraping the cells into 10 ml of PBS with a rubber policeman and pelleting by centrifugation at 300 \( \times \) g. The cells were lysed in 1 ml of 15 mm Tris-HCl (pH 8.0), 2 mm EDTA, 2 mm ethyleneglycol bis(\( \beta \)-aminoethyl ether)-N,N',N"-N"-tetracetatic acid, 2 mm benzamidine KCl, 10 \( \mu \)m aprotinin, 1.0% bovine serum albumin, 1 mm phenylmethylsulfonylfluoride, 0.5% deoxycholate, and 0.5% Triton X-100. The lysate was clarified by centrifugation at 12,000 \( \times \) g. The pellet was discarded. Rabbit anti-PLAP serum was added to the supernatant and incubated overnight at 4°C. The antigen-antibody complexes were bound to protein-A Sepharose and eluted into either SDS sample buffer (22) or isoelectric focusing sample buffer (23).

Enzyme Digestion. PI-PLC was isolated from crude Bacillus cereus phospholipase C (Sigma, type III) as described previously (24). Alkaline phosphatase was released from intact cells after washing the flask two times with 5 ml PBS and incubating at 37°C for 2 ml of 10 mm Hepes (pH 7.0), 0.25 mm sucrose, and 10 mm glucose containing 60 milliunits of PI-PLC. One unit is defined as the amount of enzyme hydrolyzing 1 \( \mu \)mol \( [2-\text{H}] \)phosphatidylinositol/min at 37°C. About 2000 milliunits of AP activity was present in each flask. The cells were pelleted and the supernatant and pellet analyzed separately. Alternatively, the cells were scraped from the flask with a rubber policeman into PBS, pelleted by centrifugation, and dissolved in 1 ml of lysis buffer as above. Sixty milliunits of PI-PLC was added and the mixture incubated at 37°C for 1 h. The samples were immunoprecipitated as above.

Assay and Purification of BeWo Alkaline Phosphatase. AP activity was determined on PI-PLC supernatants or on cell pellets dissolved in 10 mm Hepes (pH 7.0), 0.5% Triton X-100. Aliquots up to 50 \( \mu \)l were added to 200 \( \mu \)l of 0.1 mm Tris-\( \text{NaOH} \) (pH 10.2), 0.5 mm MgCl\(_2\), and 6 mm \( p \)-nitrophenyl phosphate and incubated at 37°C for 1 h. One ml of 0.2 n NaOH was added and the absorbance at 410 nm was determined. One unit is defined as the amount of enzyme that cleaves 1 \( \mu \)mol of \( p \)-nitrophenyl phosphate in 1 min at 37°C (25). Methionine labeled and unlabeled BeWo cells were extracted with butanol as described (25). AP was immunoprecipitated from the extract after 2-fold dilution with lysis buffer. Twenty thousand dpm of labeled protein was added to each unlabeled sample, and the proteins were purified by elution from SDS-polyacrylamide gels after electrophoresis. The fractions containing the labeled AP were pooled, lyophilized, and dissolved in 10 mm Hepes (pH 7.0), 0.5% Triton X-100. Protein was determined by a published method (26). Fifteen \( \mu \)g of protein was obtained from 10 T-150 flasks.

Polyacrylamide Gel Electrophoresis. SDS slab gels and tube gels were done in 10% acrylamide gels as described by Laemmli (22). Isoelectric focusing in pH 5-7 gradients was done for 9000 V h in vertical slab gels with NP-40 and urea according to the method of O'Farrell (23). Fluorograms of sodium salicylate-impregnated gels were made using Kodak X-Omat AR film with intensifying screens at \(-70^\circ\)C. Quantitation of band density was done by laser densitometry. Protein transfers from SDS gels to Millipore Immobilon-P membranes were performed as described by the manufacturer. Immunoblots with rabbit anti-AP serum (kindly given by M. Turner and A. Gurnett) were exposed to the Promega Protoblot system according to the manufacturer's instructions.

RESULTS
cDNA Sequence for the Choriocarcinoma AP. The oligonucleotide sequence and predicted amino acid sequence of the longest clone isolated from the BeWo cDNA library is given in Fig. 1. The cDNA is 2464 base pairs long and includes an open reading frame of 1596 base pairs encoding a protein of 532 amino acids. A 30-base pair 5'- and an 838-base pair 3'-untranslated region are present. A single polyadenylation signal site begins 21 base pairs from the polyadenylate tail. Comparison with the cDNA sequence for the Nagao isozyme derived from the genomic germ cell AP DNA sequence reveals >99% identity (7). There are 3 nucleotide differences in the coding region at positions 201, 977, and 1317. Nine differences are present in the 3'-untranslated region. The high degree of identity suggests that the BeWo choriocarcinoma AP is identical to the Nagao isozyme and that these cells ectopically express the gene for the Nagao isozyme rather than exhibiting enhanced expression of the normal term PLAP or the first trimester AP (tissue-nonspecific AP). The base changes probably represent allelic differences or sequencing errors between the two germ cell APs.

The protein sequence predicted from the cDNA is identical to that predicted from the AP cDNA isolated from JEG-3 cells, another choriocarcinoma cell line, except for a substitution of arginine for proline at position 478 (27). Comparison of the BeWo AP, Nagao, and PLAP predicted protein sequences reveals 2 amino acid differences between the choriocarcinoma AP and the Nagao isozyme and 11 between the choriocarcinoma AP and the PLAP isozyme (Table 1). The same 11 changes are present when comparing the Nagao sequence with the PLAP sequence. The region containing the phosphate-binding serine at position 92 is conserved in all sequences, as are the two putative N-glycosylation sites at positions 123 and 250 (Fig. 1). Of interest are two differences in the COOH terminus between the Nagao or choriocarcinoma AP and PLAP (Table 1). Both changes (proline for arginine at position 490 and glycine for glutamate at position 508) occur in the COOH terminal peptide that is removed during the modification of PLAP with PI-glycan (Fig. 1) (28). These 2 amino acid substitutions could alter the properties of the COOH terminal peptide and prevent BeWo AP (Nagao AP) from modification by PI-glycan.

Release of BeWo Alkaline Phosphatase by PI-PLC. To determine whether the Nagao isozyme is anchored on the cell surface of BeWo cells, intact cell monolayers were treated with PI-PLC. Release of a protein from the cell surface by treatment with PI-PLC has been taken as presumptive evidence that the released protein is anchored by PI-glycan (13, 29). Sixty to 65% of AP is released from the surface of BeWo cells by PI-PLC, as reported for other cell types (25, 30, 31) (Fig. 2). PI-PLC
treatment of cells removed from the culture dish with a rubber policeman and treatment with 10-fold more PI-PLC released comparable amounts of AP from the BeWo cells (data not shown). No AP was spontaneously released into the medium by untreated cells. into the medium after treatment of the cells with PI-PLC.

containing the serine phosphate acceptor; underlines, the COOH terminal peptide that is cleaved during PI-glycan addition.

by mobility from untreated enzyme (Lane 1). An M, 64,000 protein was present in each sample. A small amount of M, 61,000 protein was present in the untreated sample (Fig. 3A, Lane 1). These results confirm that the BeWo AP is released into the medium after treatment of the cells with PI-PLC. Examination of the same samples by isoelectric focusing reveals a dramatic change in the mobility of the PI-PLC-treated AP (Fig. 3B). Multiple isoforms are present in both the untreated and PI-PLC-treated AP. Pretreatment of the sample

Fig. 1. cDNA sequence for BeWo alkaline phosphatase and predicted protein sequence. The nucleotide sequence for the clone encoding the BeWo AP is given with the predicted protein sequence presented in the single-letter code below each codon. Asterisks, the potential N-glycosylation sites; double underlines, the tripeptide containing the serine phosphate acceptor; underlines, the COOH terminal peptide that is cleaved during PI-glycan addition.

by mobility from untreated enzyme (Lane 1). An M, 64,000 protein was present in each sample. A small amount of M, 61,000 protein was present in the untreated sample (Fig. 3A, Lane 1). These results confirm that the BeWo AP is released into the medium after treatment of the cells with PI-PLC. Examination of the same samples by isoelectric focusing reveals a dramatic change in the mobility of the PI-PLC-treated AP (Fig. 3B). Multiple isoforms are present in both the untreated and PI-PLC-treated AP. Pretreatment of the sample

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Table 1

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* From Millan and Manes (7).
* From Kam et al. (38), Henthorn et al. (39), and Ovitt et al. (16).
"The BeWo and Nagao sequences differ at positions 38 and 297.
"The COOH terminal amino acids at positions 490 and 508 are boxed.

Fig. 2. Release of alkaline phosphatase from BeWo cells by PI-PLC. Cell monolayers were incubated for various times in the presence or absence of PI-PLC. The cells and supernatant were separated by centrifugation. AP activity was measured in the supernatant and a Triton X-100 extract of the cell pellet as described in "Materials and Methods." The values are expressed as a percentage of the total activity (40-45 milliunits). , Pl-PLC-treated cells; , untreated cells.

Fig. 3. Electrophoresis of BeWo alkaline phosphatase released by PI-PLC. Butyrate-induced cells were labeled for 1 h with [35S]methionine. After PI-PLC treatment, the cells and supernatant were separated by centrifugation and the AP was isolated by immunoprecipitation from both the supernatant and the detergent extract of the cell pellet. A, SDS-polyacrylamide gel of the immunoprecipitation from the untreated cells (Lane 1), PI-PLC-treated supernatant (Lane 2), and PI-PLC-treated cell pellet (Lane 3). B, IEF (cathode at the bottom) of untreated cells (Lane 1), PI-PLC supernatant (Lane 2), and PI-PLC cell pellet (lane 3). The lower molecular weight band seen in A (Lane 2) is nonspecific and was present in control samples that were precipitated with rabbit preimmune serum (data not shown).

Antibody Detection of PI-glycan. Although release of the BeWo AP by PI-PLC provides strong presumptive evidence that the BeWo AP is anchored by PI-glycan, confirmation of the presence of the PI-glycan moiety was obtained by binding to the AP with an antibody specific for PI-glycan. Antibodies directed against the soluble form of the Trypanosoma VSG (anti-CRD) bind specifically to the PI-glycan moiety on proteins only after PI-PLC treatment (32). The antibodies recognize the inositol phosphate that is exposed by PI-PLC cleavage of the diacylglycerol group from PI-glycan and will not bind to the intact PI-glycan (32). The antibody to soluble Trypanosoma VSG bound to the PI-PLC-treated BeWo AP (Fig. 4, Lane 3). As expected, the anti-CRD did not bind to the untreated BeWo AP (Fig. 4, Lane 4). Both the anti-placental AP antibody and the anti-CRD antibody detected a smaller, M<sub>r</sub> 61,000, protein (Fig. 4, Lane 3). These results confirm the presence of PI-glycan on BeWo AP.

Pulse-Chase Experiments. The smaller species of AP could result from the expression of another gene for AP or could represent a newly translated, incompletely processed precursor of the M<sub>r</sub> 64,000 AP. The relationship of the M<sub>r</sub> 61,000 protein to the M<sub>r</sub> 64,000 AP was examined by pulse-chase experiments. Cells labeled for 5 min with [35S]methionine after butyrate induction, solubilized, and incubated in the presence (Lanes 1 and 3) or absence (Lanes 2 and 4) of PI-PLC. After immunoprecipitation and SDS gel electrophoresis, the proteins were transferred to Immobilon-P membranes. A radioautogram (Lanes 1 and 2) and an immunoblot with the anti-CRD antibody (Lanes 3 and 4) were done.
results of the anti-CRD-binding and pulse-chase experiments. Proteins were detected by anti-placental AP antibody. The proteins. In other experiments not shown, both M, 61,000 proteins was obtained with pulse-chase experiments does not yet have PI-glycan attached.

61,000 proteins was obtained with pulse-chase experiments containing unlabeled methionine, the more acidic protein was no longer present, but the more basic form was readily detectable. By 90 min, the label was present only in the mature, M, 64,000 proteins. In other experiments not shown, both M, 61,000 proteins were detected by anti-placental AP antibody. The results of the anti-CRD-binding and pulse-chase experiments demonstrate that the more acidic, M, 61,000 protein (Figs. 6 and 7, arrows) is a precursor of the major, more basic, M, 61,000 protein (Fig. 6, arrowhead). Additionally, the BeWo AP that is unmodified by PI-glycan is present for only a short time.

Transfection of the BeWo AP cDNA. To demonstrate conclusively that the AP encoded for by the BeWo cDNA is anchored in the cell membrane by PI-glycan, the cDNA was transfected into COS-1 cells. Seventy-two h posttransfection the cells were harvested and extracted with butanol, and the AP activity was assayed. The transfected cells contained about 50 times more AP than mock-transfected cells (1030 versus 20 milliunits). Furthermore, treatment of intact cells with PI-PLC removed 90% of the AP activity present in the total cell pellet. If the cells were labeled with [35S]methionine and the AP purified by binding to antibody against PLAP, two species of AP were detected in the cells (Fig. 8). Both species were released from the cells by treatment with PI-PLC but not by incubation with buffer alone (Fig. 8, Lanes 3 and 4). No labeled AP was detected in mock-transfected cells by the PLAP antibody (data not shown). The differences between the two species of AP made by the transfected cells were not further investigated but could represent differences in posttranslational processing such as variable oligosaccharide addition. These results demonstrate that both species do contain the PI-glycan moiety. Thus, we have definitively demonstrated, for the first time, that the choriocarcinoma AP (Nagao isoform) contains the recognition sites for the enzyme(s) that add PI-glycan to proteins despite

Evidence for a temporal relationship between the two M, 61,000 proteins was obtained with pulse-chase experiments (Fig. 7). Both proteins were labelled after a 5-min pulse with [35S]methionine. Within 10 min of incubation in medium containing unlabeled methionine, the more acidic protein was no longer present, but the more basic form was readily detectable. By 90 min, the label was present only in the mature, M, 64,000 proteins. In other experiments not shown, both M, 61,000 proteins were detected by anti-placental AP antibody. The results of the anti-CRD-binding and pulse-chase experiments

Fig. 7. Two-dimensional electrophoresis of BeWo alkaline phosphatase after pulse-chase labeling. Butyrate-induced cells were labeled with [35S]methionine for 5 min and chased for the indicated times, in minutes (ordinate), with excess cold methionine. The AP was immunoprecipitated and analyzed with two-dimensional electrophoresis. Arrows, the positions of the AP species that does not bind anti-CRD antibody (Fig. 6).

Alkaline Phosphatase Precursor without PI-glycan. In Trypanosoma, PI-glycan is present on VSG even after a 1-min pulse and no unmodified protein could be detected (33, 34). Similarly, no protein lacking the PI-glycan was detected in BeWo cells by SDS-PAGE. Because of the ability to separate isoforms of BeWo AP by isoelectric focusing, two-dimensional gels (SDS/IEF) were used to separate all species of BeWo AP in an attempt to identify a species of BeWo AP that does not contain PI-glycan. The presence of PI-glycan was determined by binding of the anti-CRD antibody to PI-PLC-treated AP. In cells continuously labeled with [35S]methionine for 3 h, a protein with a more acidic pi (Fig. 6, A and B, arrows) and less abundant than the more basic M, 61,000 protein (Fig. 6, A and B, arrowheads) is present. The anti-CRD antibody bound the major M, 61,000 protein (Fig. 6D, arrowhead) but did not bind the more acidic species of AP after PI-PLC digestion (Fig. 6D, arrow). The more acidic protein thus represents a form of BeWo AP that does not yet have PI-glycan attached.

Evidence for a temporal relationship between the two M, 61,000 proteins was obtained with pulse-chase experiments (Fig. 7). Both proteins were labelled after a 5-min pulse with [35S]methionine. Within 10 min of incubation in medium containing unlabeled methionine, the more acidic protein was no longer present, but the more basic form was readily detectable. By 90 min, the label was present only in the mature, M, 64,000 proteins. In other experiments not shown, both M, 61,000 proteins were detected by anti-placental AP antibody. The results of the anti-CRD-binding and pulse-chase experiments
cells were labeled with [35S]methionine for 3 h and harvested for treatment with choriocarcinoma AP and FLAP. Lane 4, the AP released into the medium by PI-PLC treatment. 

Lane 1, the AP present in the cell pellet after incubation buffer or with PI-PLC. The BeWo AP was purified by immunoprecipitation and with DEAE-dextran as described in “Materials and Methods.” After 72 h, the infected into COS-1 cells. cDNA for the BeWo AP transfected into COS-1 cells was recognized by polyclonal antisera to FLAP, even though 

DISCUSSION

We have presented evidence that the AP expressed by a choriocarcinoma cell line, BeWo, is identical to the product of the recently described germ cell AP gene and differs from normal term PLAP (7). This gene encodes the Nagao isozyme found in normal germ cells, testis, thymus, and some tumors of germ cell origin. A similar enzyme has not been described in either first trimester or term placenta by immunological or biochemical methods (14, 35). Additionally, translation of first trimester placental RNA failed to demonstrate a product that was recognized by polyclonal antisera to PLAP, even though the antibody cross-reacts with the Nagao isozyme (16). RNA blots of first trimester and term placenta did not reveal an RNA species of the same size as the AP RNA in choriocarcinoma cells (16). This evidence suggests that the Nagao isozyme is normally not expressed by trophoblasts. Thus, the AP synthesized by these choriocarcinoma cells is the product of a derepressed gene and not the enhanced expression of a gene normally expressed by the progenitor cells. Because seminomas express an AP gene normally expressed in their progenitor cells, germ cells, the molecular events leading to the expression of the same gene in two separate tumors, seminoma and choriocarcinoma, as a consequence of tumorigenesis, may differ substantially.

Although both seminomas and choriocarcinomas express the same AP gene, the fate of the AP may be different. Seminomas, like choriocarcinomas, have AP on their cell surface demonstrated by immunochemical techniques (36). Patients with seminomas also have high levels of tumor-derived AP in their serum (10, 11). In contrast, patients with choriocarcinomas generally have low levels of tumor AP in their serum (12). The reason for the difference in serum levels of AP between the two tumors is not clear. By the criteria of PI-PLC release and binding to anti-CRD, we have demonstrated that the BeWo AP and the AP expressed by cells transfected with the AP cDNA are anchored to the plasma membrane by PI-glycan (32). If the seminoma enzyme were similarly anchored in the membrane by PI-glycan, the secreted AP could arise from release of the seminoma AP by a PI-PLC that is present in those tumors and not expressed in choriocarcinomas, or, perhaps, the seminoma AP is more accessible to serum phospholipase D, which can also cleave the PI-glycan. Alternatively, the seminomas may express a separate, secreted species of AP. The secreted AP may be the product of another gene or the result of alternative RNA splicing as found in other enzymes that are both secreted and anchored in the membrane by PI-glycan (37).

Because only a portion of AP is released from cells, including BeWo cells, or membrane preparations by PI-PLC, others have suggested that there may be a second anchoring mechanism for AP (13, 29). If other anchoring mechanisms were present, then, the molecular events resulting in the different forms of AP may exist. The control of the different anchors could result from posttranslational modifications or from the translation of different mRNA species that arise as a product of alternative splicing or of separate genes. We were able to quantitate the amount of AP that was modified by PI-glycan utilizing our observation that PI-PLC treatment shifted the pI of BeWo AP on IEF. Analysis of the PI-PLC-treated AP by IEF demonstrated that 95% of BeWo AP is modified with PI-glycan. Moreover, binding of the BeWo AP by the anti-CRD antibody, which is specific for PI-glycan, confirmed that at least 95% of the AP contains a PI-glycan structure susceptible to PI-PLC. Thus, our results clearly show that virtually all of the BeWo AP is modified by PI-glycan, and no other anchoring mechanisms need to be postulated. However, interactions with other membrane components or the presence of an additional hydrophobic, membrane-binding site cannot be excluded by these results.

Like the Trypanosoma VSG, the modification of BeWo AP with PI-glycan occurs early in the synthesis and processing of the protein. Unlike the Trypanosoma VSG, an intermediate that was not modified by PI-glycan was identifiable by us on two-dimensional gel electrophoresis (Fig. 6). This protein appeared to be a short-lived precursor of another intermediate species that contained PI-glycan but still had only high-mannose oligosaccharides. These experiments cannot determine whether or not a COOH terminal peptide was removed from the protein that was unmodified by PI-glycan but do demonstrate that the PI-glycan modification occurs after oligosaccharide addition and that the modification is posttranslational, not cotranslational.

Tumor cells can express homologous genes such as PLAP and Nagao isozymes, both ectopically and eutopically. The regulators that determine the eutopic and ectopic expression of such highly homologous genes in tumor cells remain unknown. Knowledge of gene regulation and biosynthesis of gene products

Fig. 8. SDS-PAGE analysis of the BeWo alkaline phosphatase cDNA transfected into COS-1 cells. cDNA for the BeWo AP transfected into COS-1 cells with DEAE-dextran as described in “Materials and Methods.” After 72 h, the cells were labeled with [35S]methionine for 3 h and harvested for treatment with buffer or with PI-PLC. The BeWo AP was purified by immunoprecipitation and analyzed by SDS-PAGE. Lane 1, the AP present in the cell pellet after incubation with buffer; Lane 2, the AP present in the cell pellet after incubation with PI-PLC; Lane 3, the material from the medium of the cells incubated with buffer; Lane 4, the AP released into the medium by PI-PLC treatment.

the amino acid differences in the COOH terminus between the choriocarcinoma AP and PLAP.

Fig. 8. SDS-PAGE analysis of the BeWo alkaline phosphatase cDNA transfected into COS-1 cells. cDNA for the BeWo AP transfected into COS-1 cells with DEAE-dextran as described in “Materials and Methods.” After 72 h, the cells were labeled with [35S]methionine for 3 h and harvested for treatment with buffer or with PI-PLC. The BeWo AP was purified by immunoprecipitation and analyzed by SDS-PAGE. Lane 1, the AP present in the cell pellet after incubation with buffer; Lane 2, the AP present in the cell pellet after incubation with PI-PLC; Lane 3, the material from the medium of the cells incubated with buffer; Lane 4, the AP released into the medium by PI-PLC treatment.

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may lead to the development of improved diagnosis and therapy of tumors. The expression of AP by seminomas and choriocarcinomas provides a system to study both regulation of oncotrophic and the biosynthesis of their protein products.

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