Pst I restriction fragment length polymorphism of the human placental alkaline phosphatase gene in normal placentae and tumors

(isoenzymes/ectopic expression)

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ABSTRACT The structure of the human placental alkaline phosphatase gene from normal term placentae was studied by restriction enzyme digestion and Southern blot analysis using a cDNA probe to the gene for the placental enzyme. The DNA digests fall into three distinct patterns based on the presence and intensity of an extra 1.1-kilobase Pst I band. The extra 1.1-kilobase band is present in 9 of 27 placentas, and in 1 of these samples the extra band is present at double intensity. No polymorphism was revealed by digestion with restriction enzymes EcoRI, SmaI, BamHI, or Sac I. The extra Pst I-digestion site may lie in a noncoding region of the gene because no correlation was observed between the restriction fragment length polymorphism and the common placental alkaline phosphatase alleles identified by starch gel electrophoresis. In addition, because placental alkaline phosphatase is frequently re-expressed in neoplasms, we examined tissue from ovarian, testicular, and endometrial tumors and from BeWo choriocarcinoma cells in culture. The Pst I-DNA digestion patterns from these cells and tissues were identical to those seen in the normal ovary and term placentae. The consistent reproducible digestion patterns seen in DNA from normal and tumor tissue indicate that a major gene rearrangement is not the basis for the ectopic expression of placental alkaline phosphatase in neoplasia.

The alkaline phosphatases [orthophosphoric-monooester phosphohydrolase (alkaline optimum), EC 3.1.3.1] are a group of metalloenzymes that hydrolyze phosphate esters at a high pH optimum (pH 10–10.5). At least three gene loci exist for the human alkaline phosphatases (ALPases) including term placental ALPase (pALPase), intestinal ALPase (iALPase), and liver/kidney/bone, or tissue unspcific, ALPase (lkbALPase). The enzymes are dimers—the subunits are of 64,000–72,000 in Mr and are structurally, biochemically, and immunologically distinct. pALPase is of particular interest from both a genetic and medical standpoint because of its extensive genetic polymorphism and ectopic expression in some human cancers. The first suggestion that pALPase might be useful as a tumor marker came in 1968 when Fishman et al. (1) reported the production of a placental-like ALPase by a bronchogenic carcinoma. Subsequent studies have revealed the ectopic production of the placental isoenzyme of ALPase in a variety of malignancies (2), with a particularly high prevalence in germ cell tumors, such as seminomas (3, 4) and ovarian and uterine cancer (3, 5, 6).

The polymorphic nature of pALPase was first documented by Boyer in 1961 (7), and subsequent electrophoretic, immunologic, and genetic studies in human populations suggest there are three common alleles of the pALPase gene (8–10). These common types were designated pALPase1, pALPase2, and pALPase3, which were suggested to give rise to six common phenotypes defined by homozygotes 1:1, 2:2, and 3:3 and heterozygotes 1:2, 1:3, and 2:3. In addition, many rare alleles are responsible for a large number of electrophoretically and immunologically detectable enzyme variants (accounting for 2.5% of the population) that are believed to be heterozygotes of one of the three common alleles and one of these rare alleles (8–12).

In this study we use a full-length pALPase cDNA probe to provide evidence of allelic Pst I restriction fragment length polymorphism of the pALPase gene locus. We also compare the genomic DNA of normal placentae and several germ cell tumors by Southern blot analysis.

MATERIALS AND METHODS

Tissue Samples. Biopsies of tumor tissue and normal ovaries used for DNA isolation were immediately frozen in liquid nitrogen. Normal term placentae were obtained within 30 min of delivery, and samples of villus tissue totaling ~15 g of wet weight were excised and frozen in liquid nitrogen.

Cell Lines. The human choriocarcinoma cell line BeWo was obtained from the American Type Culture Collection (ATCC CCL98) and was grown in 50% Waymouth's MB 752/1 medium, 40% Gey's balanced salt solution, and 10% fetal calf serum with antibiotics.

Peripheral Blood Nonactivated Lymphocytes. Twenty milliliters of peripheral blood was collected in heparinized tubes.

Isolation of DNA from Tissue. Frozen pieces of tissue were crushed to powder in liquid nitrogen and lysed with 10 vol of a solution of 0.5 M EDTA, pH 8.0/0.5% (wt/vol) N-lauroylsarcosine (Sigma) containing 100 μg of protease K per ml (Sigma). Lysates were incubated overnight at 50°C. Protein was removed with three phenol extractions, and the remaining solution was extensively dialyzed in 50 mM 2-amino-2-hydroxymethyl-1,3-propanediol (Tris), pH 8.0/10 mM EDTA/10 mM NaCl. Samples were treated with 100 μg of DNase-free RNase per ml for three hr at 37°C, deproteinized by phenol/chloroform extraction, and dialyzed in TE buffer (10 mM Tris-HCl, pH 7.8/1 mM EDTA, pH 8.0) and precipitated with ethanol.

Isolation of DNA from BeWo Cells. Cells were grown in culture vessels to conflueny. Cell monolayers were washed twice with saline and harvested by scraping with a rubber policeman. DNA was extracted as above, starting with the lysis solution.

Abbreviations: ALPase, alkaline phosphatase; pALPase, placental ALPase; iALPase, intestinal ALPase; lkbALPase, liver/kidney/bone ALPase.

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Isolation of DNA from Peripheral Lymphocytes. Twenty milliliters of peripheral blood (in the presence of heparin) was washed two times with four vol of phosphate-buffered saline. The blood cells were then resuspended in 10 ml of JG buffer (50 mM Tris, pH 7.4/250 mM sucrose/5 mM MgCl2/25 mM KCl). After the addition of 30 ml of cold 0.3% Triton X-100 in JG buffer, the samples were incubated for 5 min on ice and centrifuged for 12 min at 500 x g at 4°C. This last procedure was repeated one more time, after which the nuclei were washed with JG buffer twice and treated with 1-5 mg of proteinase K in solution containing 0.3 ml of 0.5 M EDTA and 0.9 ml of 10% NaDodSO4 at 37°C overnight. DNA was denatured in phenol/chloroform extraction, extensively dialyzed in TE buffer, and then precipitated with ethanol.

Restriction Enzyme Digestion of Genomic DNA. Fifteen micrograms of DNA were digested with restriction enzymes (5 units/μg of DNA) overnight under conditions specified by the supplier (Bethesda Research Laboratories). The next day, an additional 2 units of enzyme per μg of DNA was added for 3-4 hr. Samples were ethanol precipitated overnight at -20°C and resuspended in TE buffer.

Probe. The probe used in this study was a cloned 2.7-kb pALPase cDNA prepared as described (13). The 2.7-kilobase (kb) cDNA includes ~1.7 kb of coding region and a 1083-base-pair (bp) 3’ untranslated sequence. The entire 2.7-kb cDNA was used in these studies.

32P Labeling of the Probe. The probe was labeled by incorporating deoxyctydine 5’-[α-32P]triphosphate (1,000 CPM μl-1 dCTP) using the hexadeoxynucleotide primer method described by Feinberg and Vogelstein (14). Unincorporated [32P]dCTP was removed by the spin-column procedure (15).

Southern Blot and Hybridization. Gel electrophoresis was performed on 0.7% agarose gel. Transfer of the gel to nylon paper (Genatran 45) was done according to specifications of suppliers (Plasco, Woburn, MA). After the transfer, the membrane was briefly washed in 3× standard saline citrate (SSC), (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7.0), air dried, and baked in a vacuum oven at 80°C for 1 hr. The membrane was saturated in 3× SSC buffer, placed in a plastic bag containing a prehybridization solution consisting of 3× SSC, 5× Denhardt’s solution (0.1% wt/vol Ficoll/0.1% wt/vol polyvinylpyrrolidone/0.1% wt/vol bovine serum albumin), 1 mM sodium pyrophosphate, 4.8% dextran sulfate, 1% NaDodSO4, 50% formamide, and 100 μg of denatured salmon sperm DNA per ml. Prehybridization was done at 42°C for 12 hr.

The radiolabeled probe (~0.1 μg) was then added to the blot and the hybridization was done for 20-36 hr at 42°C with gentle rocking. The membrane was washed in 2× SSC/0.1% NaDodSO4 at room temperature for 20 min, and then three times in 0.1% NaDodSO4/0.1× SSC at 65°C for 1 hr, each wash occurring in a shaking water bath. Autoradiography was done at ~70°C using a single intensifying screen for 12 hr to 5 days.

Starch Gel Electrophoresis. Placental samples were extracted with 1-butyl alcohol, and aliquots taken from the aqueous phase were electrophoresed in starch gels as described (16). ALPase was visualized by gently shaking the gels at room temperature in 100 ml of 0.06 M borate buffer (pH 9.7) containing 50 mg of β-naphthyl phosphate, 120 mg of MgSO4·7H2O, and 50 mg of fast blue B (o-dianisidine) salt. After development, the gels were fixed in a 50% methanol/10% acetic acid solution.

Solid-Phase Immunoenzyme Assay. The solid-phase immunoenzyme assay was described by McLaughlin et al. (17). The monoclonal antibodies used in the study were AAP1, which is specific for the fetal and adult forms of iATPase; H17E2, which recognizes pALPase and pALPase-like enzymes; and H317, which reacts only with pALPase. The AAP1, H1702, and H317 monoclonal antibodies were provided by D. Tucker (Imperial Cancer Research Fund Laboratories, London).

RESULTS

Samples of human genomic DNA from 27 normal term placentae were digested with the restriction enzyme Pst I and subjected to Southern blot analysis as described. The restriction enzyme Pst I was chosen for its ability to cleave the pALPase gene at specific sites as demonstrated by a pALPase cDNA restriction map. The pALPase cDNA sequence has three restriction sites for the Pst I enzyme (13, 17, 18). Southern blots show that the 27 samples of DNA fall into three distinct patterns of digestion, consisting of five or six restriction fragments ranging in size from 0.7 to 3.5 kb. Summarized results of these experiments are presented in Fig. 1. Lane A is representative of a type Pst I digestion. This designates the genomic composition of two alleles that both lack the extra or polymorphic Pst I site. Lane B presents a type Pst I* digestion consisting of the same five bands (from 0.7 kb to 3.5 kb) as the Pst I digestion plus an extra band at 1.1 kb. Lane C presents a Pst I digestion in which the 1.1-kb band is present at approximately twice the intensity seen in type Pst I*.

The distribution of genotypes in a human population, based on Southern blot analysis, is presented in Table 1. In 18 of the 27 digests of placental DNA, the 1.1-kb restriction fragment is absent; the 1.1-kb band is present in the other 9 samples and is present at approximately double the intensity in 1 sample.

Nine samples of placental DNA that contained all of the Pst I type polymorphisms were also subjected to complete digestion with the restriction enzymes EcoRI, BamHI, Smal, and Sac I. In no case did the digestion pattern resulting from these enzymes show any polymorphism. The result of Pst I and EcoRI digestion on the same group of five samples is shown in Fig. 2. This suggests that the extra Pst I site is likely to be the result of a small or point mutation.

Neoplasms that express the placental isozyme were also analyzed; there were two endometrial tumors, four ovarian tumors, one seminoma, and the BeWo choriocarcinoma cell

![Fig. 1. Southern blot analysis of human genomic DNA. Lane A, typical Pst I* digestion pattern with five restriction fragment bands. Lane B, Pst I digestion pattern exhibiting six restriction fragment bands including an extra band at 1.1 kb. Lane C, Pst I* digestion pattern where the extra 1.1-kb Pst I fragment is present in approximately twice the intensity seen in type Pst I*.](image-url)
Table 1. Distribution of Pst I restriction fragment patterns in human DNA from normal and neoplastic cells

<table>
<thead>
<tr>
<th></th>
<th>Pst I−−</th>
<th>Pst I++</th>
<th>Pst I++</th>
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</thead>
<tbody>
<tr>
<td>Placenta</td>
<td>18</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>Others</td>
<td>3 ovarian tumors</td>
<td>BeWo cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 seminoma</td>
<td>2 endometrial tumors</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 normal ovary</td>
<td>1 ovarian tumor</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>23 (64%)</td>
<td>12 (33%)</td>
<td>1 (3%)</td>
</tr>
</tbody>
</table>

Pst I-digested genomic DNA from all sources was grouped according to the appearance and density of the extra 1.1-kb fragment in Southern blot autoradiograms. Pst I−−, Pst I++, and Pst I++ designate the genomic composition of paired alleles containing either one extra Pst I site on each allele, one allele lacking the extra Pst I site, or both alleles lacking the Pst I site, respectively. The distribution of the Pst I restriction fragment patterns in 27 placentae and the distribution of the Pst I restriction fragment pattern in 4 ovarian tumors, 2 endometrial tumors, 1 seminoma, 1 normal ovary, and the BeWo cell line are shown.

The eight tumors or tumor cell lines and the normal ovary were shown to have the same types of digestion patterns as placentae. The tumors were equally divided between those having the extra 1.1-kb band and those not having the band. Furthermore, Southern blot analysis of ovarian tumor DNA and peripheral blood lymphocyte DNA from the same patient revealed identical digestion patterns (Fig. 3), indicating that no major gene rearrangement is necessary for the re-expression of pALPase in neoplasia.

The gene frequencies for the Pst I− and Pst I+ alleles were computed from the genotype ratios presented in Table 1. The

DISCUSSION

The data presented here provide evidence of a Pst I restriction fragment length polymorphism of the human pALPase gene locus. Pst I digestion of total genomic DNA from placentae results in a reproducible pattern consisting of either five or six bands upon hybridization with a full-length 2.7-kb cDNA probe.

To date, the nucleotide sequences of four different pALPase cDNA molecules have been published (13, 18, 19). The cDNA reported by Kam et al. (13) was produced from a placenta of unknown pALPase phenotype. Millan (19) has described a type 1 cDNA clone, and Henthorn et al. (18) have described both type 1 and type 3 cDNA clones. The clones

Table 2. Comparison between electrophoretic phenotype and Pst I genotype in normal placentae

<table>
<thead>
<tr>
<th>Electrophoretic pattern</th>
<th>Pst I−−</th>
<th>Pst I++</th>
<th>Pst I++</th>
</tr>
</thead>
<tbody>
<tr>
<td>PL 1</td>
<td>6</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>PL 2</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PL 2-1</td>
<td>11</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>PL 3-1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

pALPase was phenotyped by starch gel electrophoresis. DNA from the same placenta was classified by the presence or absence of the extra 1.1-kb Pst I digestion band by Southern blot analysis using pALPase cDNA probe.
are all quite similar but exhibit nucleotide substitutions at several positions that in some cases lead to amino acid changes at the corresponding positions. Unaffected by these substitutions are the three Pst I restriction endonuclease sites that are present at the corresponding regions on all four clones. Therefore, based on the restriction map of the cDNA clone, four restriction fragments would be expected from Pst I digestion. The extra band seen in the blots is the result of an additional Pst I recognition site located in the noncoding flanking or intronic regions of genomic DNA.

The extra band at 1.1 kb appears to be the result of genetic polymorphism. In several independent experiments the band was unstable and reproducible in only one of two hybridization autoradiograms, with the homozygote Pst I− making up 64% of the samples tested. The heterozygote Pst I+ occurred in 33% of the samples, whereas Pst I+ samples accounted for only 3% of the population.

The polymorphism is probably due to a point mutation leading to the creation or destruction of the extra Pst I site. This view is consistent with the results of studies on the β-globin locus that have shown that at least 1 in 100 base pairs varies polymorphically (20). A comparison of the restriction fragment patterns and starch gel electrophoresis-defined phenotype for each placenta (Table 2) reveals no direct relationship between restriction fragment pattern and any phenotype. This lack of correlation indicates that the restriction fragment length polymorphism is in one of the Pst I sites located in the noncoding regions of the pALPase gene. There does, however, appear to be a higher likelihood of those placenta expressing type I pALPase to be Pst I+. The common explanation for the diversity of the ALPases is duplication and mutation of a common ancestral gene.

We believe that by performing hybridization and washing under conditions of high stringency (42°C hybridization and three 1-hr washes at 65°C) we have reduced the possibility of cross-hybridization with other genes in the ALPase family. The nucleotide sequence of a cDNA clone of the human lbkALPase shows 52% homology to pALPase (21). Because the melting point of duplex DNA is reduced by 1°C with an increase of 1% in the number of mismatch base pairs (22), the 48% mismatch between lbkATPase and pALPase should exclude detection of lbkATPase genes with the pALPase cDNA under these conditions. In fact, Kam et al. (13) failed to detect mRNA in human liver with the same pALPase cDNA probe used in the present study. There is a greater likelihood of cross-hybridization between the pALPase probe and the iALPase gene based on the 88% nucleotide-sequence homology in the protein-coding regions of iALPase and pALPase cDNA molecules (23). However, the pALPase probe used in this study has only shown weak hybridization with mRNA from human small intestine under conditions of moderate stringency by RNA blot hybridization (24). In the present study, the bands in the Southern blots used to demonstrate the polymorphism are of high intensity, suggesting that these bands are not the result of weak cross-hybridization with the iALPase gene (Figs. 1–3). Although the use of high-stringency conditions greatly reduces cross-hybridization, the possibility that restriction fragments from the iALPase gene contribute to the digestion pattern cannot be completely excluded.

It is apparent from the studies done on seminoma and tumors of the ovaries and endometrium that no major genetic rearrangement is occurring involving the pALPase locus in ectopic expression. The eight tumors studied showed the same restriction fragment pattern and relative polymorphic distribution as those of placental tissue; additionally, the DNA restriction pattern of peripheral lymphocytes did not differ from that of the ovarian tumor in a patient. Further-

more, other restriction enzymes—EcoRI, Sma I, BamHI, and Sac I—failed to detect a polymorphism either in normal placenta or tumor tissue. If a genetic transposition of the pALPase gene were required for its ectopic expression in tumor, one would expect to see some difference in the restriction pattern between normal and neoplastic tissue. The lack of global rearrangement suggests an alternative mechanism is responsible for the expression of pALPase isoenzymes in neoplasia.

Note Added in Proof: Southern blot analyses were performed on the same Pst I human genomic DNA digests using an iALPase cDNA probe (generously provided by Dr. Harry Harris, Department of Genetics, University of Pennsylvania). The results show that the intestinal probe hybridizes weakly to the polymorphic 1.1-kb fragment in contrast to the strong hybridization observed with the placental probe, indicating that the polymorphism is derived from the pALPase gene.

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