Characterization of KB Cell Alkaline Phosphatase

EVIDENCE OF SIMILARITY TO PLACENTAL ALKALINE PHOSPHATASE*

(Received for publication, September 25, 1975)

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The alkaline phosphatase from KB cells was purified, characterized, and compared to placental alkaline phosphatase, which it resembles immunologically. Two nonidentical monomeric subunits of the KB phosphatase were found. The two subunits, which have apparent molecular weights of 64,000 and 72,000, can be separated on polyacrylamide gels containing sodium dodecyl sulfate. The $M_r = 64,000$ KB subunit appears to be identical in protein structure to the monomer of placental alkaline phosphatase. The $M_r = 72,000$ KB subunit, while differing in the $NH_2$-terminal amino acid, appears also to be very similar to the placental alkaline phosphatase monomer. Both KB phosphatase subunits bind [32P]phosphate, and bind to Sepharose-bound anti-placental alkaline phosphatase. Native KB phosphatase is identical to the placental isozyme in isoelectric point, pH optimum, and inhibition by amino acids, and has a very similar peptide map. The data presented support the hypothesis that the $M_r = 64,000$ KB phosphatase subunit may be the same gene product as the monomer of placental alkaline phosphatase. This paper strengthens the evidence that the gene for this fetal protein, normally repressed in all cells but placenta, is derepressed in the KB cell line. In addition, this paper presents the first structural evidence that there are two different subunit proteins comprising the placental-like alkaline phosphatase from a human tumor cell line.

The placental form of alkaline phosphatase (EC 3.1.3.1) is normally produced only by the placenta (2). However, a placental-like alkaline phosphatase has been reported in human and mouse neoplasms (3, 4) and in HeLa cells (5). Other fetal proteins are also produced by some tumor cells (6). It has been hypothesized that the presence of specific fetal proteins in tumor cells results from derepression of the genes for those embryonic proteins during or after neoplastic transformation (7).

In order to study derepression and regulation of the genes for fetal proteins in tumor cells, it is first necessary to show the identity of the specific fetal proteins from normal and tumor cells. The protein structure of purified alkaline phosphatase from a primary lung tumor has been shown to be very similar to that of placental alkaline phosphatase by several structural criteria (8). But studies of gene regulation require the demonstration of identity of this gene product in a tumor cell line. We chose to study the placental-like alkaline phosphatase of KB cells because of the wide literature on this cell line and because of its nonplacental origin.

The KB cell line, derived from a nasopharyngeal tumor, is an important and widely used human tumor cell line (9). Studies of the alkaline phosphatase of KB cells (10) have concluded that two forms of KB alkaline phosphatase exist, one of which is heat-stable and is in this respect similar to placental alkaline phosphatase. The molecular and immunologic properties of the KB cell alkaline phosphatase have not been characterized, thus it has not been possible to assess whether two forms actually exist, and if so, the extent of their correspondence to placental alkaline phosphatase.

This paper presents the purification and characterization of the alkaline phosphatase from KB cells. The results of this study are of special interest because the KB cell alkaline phosphatase contained two nonidentical subunit proteins. All alkaline phosphatases previously characterized have had subunits of identical molecular weight (11, 12). One of the subunits of KB cell alkaline phosphatase may be identical to the subunit of placental alkaline phosphatase; the other KB phosphatase subunit is very similar to the placental subunit but differs in at least one amino acid. The two nonidentical KB phosphatase subunits may be controlled by allelic genes, such as those proposed by Robson and Harris (13) to account for electrophoretic variants of placental alkaline phosphatase. If so, this paper presents the first structural evidence characterizing two such variants.

EXPERIMENTAL PROCEDURE

Cell Culture—All culture media, sera, and antibiotics were from Grand Island Biological Co. unless otherwise noted. KB cells ob-
KB Alkaline Phosphatase

The abbreviations used are: MEM, Minimum Essential Medium; PAS, periodic acid-Schiff; Na dodecyl-SO₄, sodium dodecyl sulfate; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; Na dodecyl-SO₄; 2621 KB Alkaline Phosphatase; DEAE-Sephadex in 10 mM Tris/0.1 M NaCl/0.1% Triton X-100.

The immunoenzymatic assay for placental alkaline phosphatase has been described (2). After a specific reaction between enzyme and anti-enzyme γ-globulin, the complex is precipitated with an anti-γ-globulin. Enzyme activity in the supernatant is determined by incubating the gel with p-nitrophenyl phosphate. The yellow enzyme band can be marked with razor cuts prior to staining with Coomassie blue.

Nondenaturing Polyacrylamide Gel Systems—The discontinuous buffer system of Laemmli (93) with 0.1% Na dodecyl-SO₄, was used to examine the subunits of alkaline phosphatase and to determine their molecular weights. Bovine serum albumin (Mₙ = 68,000), tropomyosin (Mₙ = 36,000) and chymotrypsigen A (Mₙ = 95,000) were used as molecular weight standards. Molecular weights were estimated from a graph of the log of the relative mobility versus the molecular weights of the standards (24).

The gel system of Swank and Munkres (25) was also used to examine the subunits of alkaline phosphatase. Before electrophoresis in this system in gels containing 4.6% acrylamide, the KB phosphatase was reduced and carboxymethylated (26).

Weber-Obshorn (27) polyacrylamide gels (5% acrylamide, cylindrical tube gels) were used to separate enough of the KB subunit proteins for NH₄-terminal amino acid sequencing. The gels were frozen, and a series of 1-mm slices was made in the region where the proteins were expected to be. Each gel slice was eluted separately into 5 mM NaHCO₃ containing 0.05% Na dodecyl-SO₄. A small aliquot of each gel slice was run on a Laemmli slab gel for identification. Fractions containing one pure subunit were pooled, concentrated, and centrifuged 30 min at 10,000 × g to remove any small pieces of acrylamide.

NH₄-terminal Amino Acid Sequencing—NH₄-terminal sequence analysis was done according to Weiner et al. (28). This method combines stepwise Edman degradation of the protein with thin layer chromatography of the dansylated NH₄-terminal amino acid residues on two-sided polyamide sheets. Identification of each amino acid was made by co-chromatography of the sample on a dansylated standard on one side of the sheet and the sample alone on the other side.

Peptide Mapping—The proteins were reduced and carboxymethylated according to Sawyer et al. (29). Native KB alkaline phosphatase was used, as well as the two subunits of KB phosphatase separated on Weber-Obshorn gels. The pure KB subunit proteins were dialyzed extensively versus 10 mM Tris with DEAE-cellulose to remove Na dodecyl-SO₄. Carboxymethylated protein, 300 µg (enough for two maps) was dissolved in 1 ml of 0.1 M ammonium bicarbonate buffer, pH 8.2, at 57°C. Trypsin ( Worthington, L-1-tosylamido-2-phenyl) ethyl chloromethyl ketone treated) was added in 2-ml aliquots initially and after 1 hour from a stock solution of 2 µg/ml. After 4 hours, the digested protein was lyophilized. The peptide mixture was spotted on plates (20 × 30 cm) of thin layer silica gel on plastic (Fastman No. 8001). Chromatography was done in 1-butanol/pyridine/acetic acid/water (75/45/15/10), a system which was developed specifically for placental alkaline phosphatase. Thin layer chromatographic migration of peptides was achieved with peptides of either the separated KB subunits or the native KB enzyme. Therefore, a one-dimensional map was done with electrophoresis alone. Peptides were electrophoresed in 8.8% formic acid at 300 volts for 2 hours 15 min at 4°C. Peptides of the two separated KB phosphatase subunits did not migrate in the electrophoresis step, possibly due to being Na dodecyl-SO₄.
Therefore, the peptide mapping was done with native KB phosphatase (mixture of the two nonidentical subunits) and with placental alkaline phosphatase. After electrophoresis, the thin layer plates were dried, and sprayed with 2 mg of fluorescamine (Fluram, Roche Products) in 30 ml of acetonitrile per plate. Peptide spots were visualized with ultraviolet light and traced directly on the plate. The plate was later sprayed with ninhydrin/cadmium.

**Affinity Chromatography—Anti-placental alkaline phosphatase γ-globulin and nonspecific sheep y-globulin were bound to cyanogen bromide-activated Sepharose 4B (Pharmacia) as in Cuatrecasas et al. (29). Purified KB phosphatase (10 μg) was washed into a column of 6 ml of the Sepharose in 0.2 M Tris/0.5 M NaCl, pH 8.0. The column was washed with 12 ml of the Tris buffer, then with 10 ml of 5 M urea/1 M NaCl. Fractions from the column were checked for alkaline phosphatase activity and were electrophoresed in a Laemmli slab gel.

**[^3P]Phosphate Binding—Purified KB and placental alkaline phosphatases were labeled with carrier-free H[^32]PO_4 (New England Nuclear) following the technique of Milstein (30). Membrane-enriched fractions from KB cells and from HeLa cells were also labeled. The membrane fractions were prepared as in Brunette and Till (31), except that in the two-phase system 20 mM NaHCO_3, pH 8.0, was used instead of 0.22 M phosphate buffer, and 10 mM MgCl_2 was substituted for 10 mM ZnCl_2 (because Zn^{2+} can inhibit alkaline phosphatase activity).

The phosphate samples were labeled by incubation with[^3P]phosphate at pH 5.0 for 10 min at 0°C. For the purified KB and placental alkaline phosphatases, 5 μg and 0.3 μg were incubated with 20 μCi and 5 μCi of[^3P]phosphate, respectively. For the crude membrane fractions, 0.2 to 0.4 ml of packed cells were homogenized, and the protein collected from the interface of the two-phase system was incubated with 100 μCi of[^3P]phosphate.

The[^3P]labeled phosphate and membrane fractions were washed with ether after the washes with acid acetone, and dried with N_2. The samples were dissolved in Laemmli sample buffer, heated 10 min at 90°C, and electrophoresed in a Laemmli slab gel. After electrophoresis, the gel was fixed for 3 min in 10% trichloroacetic acid and washed overnight in 25% methanol/5% acetic acid. After staining with Coomassie blue (1 hour) and destaining (4 hours) the gel was dried down between sheets of dialysis membrane (Hoeffer Scientific Inc.) and was incubated with x-ray film. Part of each gel, with duplicate samples, was not dried down, but protein bands were sliced out and dissolved with H_2O_2, and radioactivity was determined by scintillation counting.

**Neuraminidase Treatment—Alkaline phosphatase preparations were treated with neuraminidase (Worthington NEUP; 1.5 units/mg) as in Mori and Hollands (32). After dialysis against distilled water, the samples were concentrated in Amicon B15 Minicon concentrators, mixed with the appropriate sample buffers, and analyzed by native and denaturing polyacrylamide gel electrophoresis.

**RESULTS**

**Alkaline Phosphatase Activity of Cell Lines**—The specific antisera against placental alkaline phosphatase can distinguish placental alkaline phosphatase activity from the alkaline phosphatases made by other organs, e.g., liver or kidney (2). Several human tumor cell lines were homogenized and tested immunologically for placental alkaline phosphatase activity (Table I). Of the cell lines tested, HeLa, KB, and ChaGo cells had high alkaline phosphatase activity, 95 to 100% of which reacted with the specific anti-placental alkaline phosphatase antisera. While identical immunologic reactivity does not necessarily imply identity of protein structure, these results were suggestive of the production of a specific placental protein by a number of tumor cell lines in culture. Therefore, the placental-like phosphatase from a tumor cell line (KB cells) was compared to the normally produced placental alkaline phosphatase in order to determine the degree of structural similarity of the two proteins.

**Purification of KB Phosphatase**—Alkaline phosphatase was purified from a membrane-enriched fraction of KB cells. The KB alkaline phosphatase activity eluted from a DEAE-cellulose column as a single peak (Fig. 1). On a Sephadex G-200 column, 97% of the alkaline phosphatase eluted in the void volume (Fig. 2). The peak enzyme fraction from the Sephadex G-200 column was further purified by isoelectric focusing (Fig. 3). The KB alkaline phosphatase has an isoelectric point of pH 4.3 (the same as placental alkaline phosphatase).

**Polyacrylamide Gel Analysis of KB Alkaline Phosphatase**—In a nondenaturing gel system (22) without Triton X-100, the major portion of the KB alkaline phosphatase activity does not migrate into the gel. With 0.1% Triton X-100 in the sample and gel system, the KB phosphatase migrates as two bands of enzyme activity corresponding to two protein bands (Fig. 4) which appear to be of unequal quantity by Coomassie blue staining. When each of these two enzyme bands are sliced out of the nondenaturing gel, ether-extracted to removed Triton X-100, and electrophoresed separately in a Laemmli gel (containing Na dodecyl-SO_4), the same two nonidentical subunit proteins are seen in the eluates from both of the enzyme slices.

The small fraction (about 3%) of KB alkaline phosphatase
KB Alkaline Phosphatase

Fig. 2. Sephadex G-200 chromatography of KB phosphatase. •, alkaline phosphatase activity (µmol/ml/min); O, protein (mg/ml). The column (2.5 × 75 cm) was run with a pressure head of 17 cm, which gave a flow rate of 0.47 ml/min. The fraction size was 2.6 ml per tube. The large peak, 97% of the alkaline phosphatase activity, elutes in the void volume of the column. Void volume, fractions 1 to 65, was determined with blue dextran. Fractions 50 to 70 were pooled for further purification by isoelectric focusing.

Fig. 3. Isoelectric focusing of the KB alkaline phosphatase. •, alkaline phosphatase activity (µmol/ml/min); O, pH of fractions. Focusing was done at 4° in a 110-ml column, with ampholytes in the range of pH 3.0 to 6.0, for 40 hours. Fractions were pumped out of the column with a peristaltic pump and assayed immediately for pH and enzyme activity.

which was retarded by the Sephadex G-200 column (Fractions 81 to 95 in Fig. 2) does migrate into nondenaturing gels without Triton X-100, where it has the same mobility as placental alkaline phosphatase (0.377). However, this fraction has the same two nonidentical subunits in Na dodecyl-β-SO₄ gels as does the major portion of the KB alkaline phosphatase.

On electrophoresis in denaturing polyacrylamide gel, the purified KB phosphatase is seen to have two protein subunits (Fig. 5). The apparent molecular weights of the two KB subunit proteins are 64,000 and 72,000. (The apparent molecular weight of placental alkaline phosphatase subunit is 64,000.) Enzyme activity of the subunits cannot be determined directly, since Na dodecyl-β-SO₄ destroys all alkaline phosphatase activity.

Fig. 4. Polyacrylamide gel electrophoresis with 0.1% Triton X-100. Purified KB alkaline phosphatase (left) and purified placental alkaline phosphatase (right) were electrophoresed in 7.5% acrylamide gels in the discontinuous buffer system of Davis, with 0.1% Triton X-100 in all solutions. Bands of alkaline phosphatase activity, visualized by incubating the gels with p-nitrophenyl phosphate, corresponded to the Coomassie blue-staining protein bands shown in the photograph.

Fig. 5. Na dodecyl-β-SO₄-polyacrylamide gel electrophoresis of KB alkaline phosphatase in a gel containing 10% acrylamide, with the Laemmli buffer system. Coomassie blue staining of the KB phosphatase (bottom) shows two protein bands. A densitometer scan of the Coomassie blue-stained gel is shown in A. The origin of the gel is indicated TOP and (-) in the figure. Molecular weights of the two proteins were estimated from a graph of molecular weights versus the log of the mobilities of standard proteins electrophoresed on the same gel. The faster band (right) has an apparent \( M_t \) of 64,000; the slower band (arrow) has an apparent \( M_t \) of 72,000. The \( M_t = 64,000 \) band accounts for 53% of the total area of the two bands. B shows a densitometer scan of PAS staining of KB phosphatase run in another part of the same gel, sliced out, and stained as described under "Experimental Procedure." The \( M_t = 72,000 \) band contains 66% of the total area of the PAS-stained bands.
In order to minimize the possibility that these results were explainable by aggregation of the protein in the Laemmli (Na dodecyl-SO₄) gel system, the KB phosphatase was reduced and carboxymethylated, and electrophoresed in polyacrylamide gels containing 6 M urea and 0.1% Na dodecyl-SO₄. Under these conditions the two subunit proteins were still present, and the faster protein co-migrated with the placental alkaline phosphatase monomer (Fig. 6).

Separation of Subunits—In order to study the structure of the two KB phosphatase subunits, it was necessary to separate them into entirely pure fractions. Separation of the two subunits was accomplished by slicing frozen cylindrical Na dodecyl-SO₄ gels, and eluting the slices separately (Fig. 7). Fractions containing a single subunit were pooled, centrifuged to remove any particulate acrylamide, and dialyzed versus DEAE-cellulose to remove Na dodecyl-SO₄.

Characterization of KB Phosphatase—The NH₂-terminal amino acid sequences of the separated KB subunits are shown in Table II. The Mᵣ = 64,000 KB subunit has the same NH₂-terminal sequence as placental alkaline phosphatase. The Mᵣ = 72,000 KB subunit differs in the NH₂-terminal amino acid only, having phenylalanine instead of isoleucine. Purified liver alkaline phosphatase, in contrast, has a quite different NH₂-terminal sequence (8).

Peptide mapping was done in order to compare the entire primary structure of the KB and placental alkaline phosphatases. Single dimensional mapping was done because bound Triton X-100 was not completely removed by the repeated ether extractions and interfered with chromatography of the peptides. The one-dimensional map of the tryptic peptides of the mixture of two KB subunit proteins was compared to a map of tryptic peptides of placental alkaline phosphatase which were also electrophoresed but not chromatographed (Fig. 8). All of the peptide spots of the mixture of two KB subunits corresponded very closely to the spots of placental alkaline phosphatase peptides. Small variations such as the resolution of one large spot into two smaller spots, and small differences in distance of spots from the origin, are seen in the same protein mapped on different days. Thus, the KB and placental alkaline phosphatase spots correspond as closely as can be expected given the variability due to the technique. There was one extra spot present in the placental alkaline phosphatase peptide map which was not in the KB map. The extra placental alkaline phosphatase peptide spot was fainter than the others and was unresolved in five out of fifteen maps of placental alkaline phosphatase.

Both of the KB subunit proteins stain with PAS reagent, as does placental alkaline phosphatase, indicating that all are glycoproteins. Interestingly, the Mᵣ = 72,000 KB subunit contains more oxidizable carbohydrate than the Mᵣ = 64,000 subunit (Fig. 5), which may account for its slower mobility on Na dodecyl-SO₄ gels (and larger apparent molecular weight).

Neuraminidase treatment, which removes sialic acid groups, decreases the mobility of the KB alkaline phosphatase bands on acrylamide gels without Na dodecyl-SO₄ (Table III); the mobility of placental alkaline phosphatase is also decreased. The mobility of both KB subunit proteins on Laemmli gels (with Na dodecyl-SO₄) is increased slightly by neuraminidase treatment (Table IV). The mobility of placental alkaline phosphatase on Laemmli gels is also increased slightly by neuraminidase treatment.

Enzyme Activity of KB Phosphatase Subunits—The ques-
spots correspond as closely to the KB peptide spots as the spots of two KB phosphatase subunits proteins were done as described under “Experimental Procedure.” The peptides were spotted on a thin layer chromatography plate (circle with dot at far left) and electrophoresed in one dimension. Peptide spots were visualized with fluorescamine and ultraviolet light, and traced directly onto the thin layer chromatography plate. Except for the faint peptide (dotted line) at the far right of the placental alkaline phosphatase map, the placental alkaline phosphatase peptide spots correspond as closely to the KB peptide spots as the spots of two placental alkaline phosphatase preparations mapped at different times correspond to each other.

**TABLE III**

**Electrophoretic mobility of neuraminidase-treated alkaline phosphatases**

KB and placental alkaline phosphatase were treated with neuraminidase as described under “Experimental Procedures.” The untreated and desialized enzymes were electrophoresed in polycrylamide gels containing 0.1% Triton X-100 (without Na dodecyl-SO₄). Phosphatase activity was visualized with the enzyme substrate. Mobilities were determined relative to bromophenol blue tracking dye.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Mobility Difference in mobility</th>
</tr>
</thead>
<tbody>
<tr>
<td>KB Slow band</td>
<td>0.066</td>
</tr>
<tr>
<td>Desialized slow band</td>
<td>0.040</td>
</tr>
<tr>
<td>Fast band</td>
<td>0.119</td>
</tr>
<tr>
<td>Desialized fast band</td>
<td>0.100</td>
</tr>
<tr>
<td>Placental alkaline phosphatase</td>
<td>0.307</td>
</tr>
<tr>
<td>Desialized placental alkaline phosphatase</td>
<td>0.244</td>
</tr>
</tbody>
</table>

**TABLE IV**

**Mobility of neuraminidase-treated phosphatase in Na dodecyl-SO₄ gels**

Neuraminidase-treated alkaline phosphatases were electrophoresed in the Laemmli polyacrylamide gel system. Mobilities were determined relative to bromophenol blue tracking dye.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Mobility</th>
<th>Difference in mobility</th>
</tr>
</thead>
<tbody>
<tr>
<td>KB M₁ = 72,000 subunit</td>
<td>0.314</td>
<td>0.007</td>
</tr>
<tr>
<td>Desialized subunit</td>
<td>0.321</td>
<td></td>
</tr>
<tr>
<td>M₂ = 64,000 subunit</td>
<td>0.343</td>
<td>0.012</td>
</tr>
<tr>
<td>Desialized subunit</td>
<td>0.355</td>
<td></td>
</tr>
<tr>
<td>Placental alkaline phosphatase</td>
<td>0.343</td>
<td>0.012</td>
</tr>
<tr>
<td>Desialized placental alkaline phosphatase</td>
<td>0.355</td>
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</table>

**Fig. 8**. Peptide map of placental alkaline phosphatase (PAP) and KB alkaline phosphatase. Tryptic peptide mapping of placental alkaline phosphatase and of the mixture of two KB phosphatase subunits proteins was done as described under “Experimental Procedure.” The peptides were spotted on a thin layer chromatography plate (circle with dot at far left) and electrophoresed in one dimension. Peptide spots were visualized with fluorescamine and ultraviolet light, and traced directly onto the thin layer chromatography plate. Except for the faint peptide (dotted line) at the far right of the placental alkaline phosphatase map, the placental alkaline phosphatase peptide spots correspond as closely to the KB peptide spots as the spots of two placental alkaline phosphatase preparations mapped at different times correspond to each other.

**Fig. 9**. [³²P]Phosphate labeling of alkaline phosphatases. Autoradiography procedures are given under “Experimental Procedure.” [³²P]-labeled alkaline phosphatases were electrophoresed in a Laemmli slab gel (containing Na dodecyl-SO₄). The gel was dried down and incubated with x-ray film. The developed x-ray films were scanned on the densitometer. The origin of each gel is marked TOP (−) on the scans. The samples labeled with [³²P]phosphate were: KB, a membrane-enriched preparation from a single cloned KB cell line; PAP, purified placental alkaline phosphatase; HeLa, a membrane-enriched preparation from HeLa cells. Placental alkaline phosphatase and HeLa cells have one [³²P] binding band with the same relative mobility. KB cells have two [³²P] binding bands; one with the same mobility as placental alkaline phosphatase, the other slightly slower.

**Antigenicity of KB Phosphatase Subunits**—The native KB enzyme was applied to a column of Sepharose bound anti-placental alkaline phosphatase. No enzyme activity was eluted from the column by washing with 0.2 M Tris/0.5 M NaCl, pH 8.0. Enzyme activity was eluted by washing the column with 8 M urea/1 M NaCl. Only 25% of the enzyme activity applied to the column was recovered, and this eluted in a single 1-ml fraction containing the front of the urea wash. The loss of enzyme activity is probably due to the 8 M urea, which in control experiments inhibits 70 to 80% of the enzyme activity of placental alkaline phosphatase after 24 hours. A control protein, human transferrin, was not retained by the Sepharose anti-placental alkaline phosphatase column. In another control, placental alkaline phosphatase did not bind to a column of Sepharose bound nonspecific sheep γ-globulin.

Electrophoresis of the fractions from the affinity chromatography column in a Na dodecyl-SO₄-acrylamide gel showed no proteins eluted by the wash with Tris buffer. Many protein bands, including two at the mobilities corresponding to the two nonidentical KB subunits, were present in the first fraction eluted by the urea wash. The other bands probably represent portions of γ-globulins washed off the column by 8 M urea.

**Functional Properties of KB Phosphatase**—Data on the enzyme activity of the KB alkaline phosphatase show that it is similar in several respects to and different in one respect from placental alkaline phosphatase. Ninety-eight to one hundred percent of both KB and placental alkaline phosphatase reacted with the specific antiserum to placental alkaline phosphatase.

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Fig. 10. Heat stability of KB and placental alkaline phosphatase. ○ and △, KB alkaline phosphatase; ● and □, placental alkaline phosphatase. For each time point 48 ng of KB alkaline phosphatase or 30 ng of placental alkaline phosphatase were incubated at the given temperature (56°C or 65°C) in 0.1 ml of 0.1 M Tris buffer with 0.5 mM MgCl2, pH 7.4, or in 0.1 ml of 0.1 M monothanolamine buffer with 0.5 mM MgCl2, pH 11.0. At each time point, the samples taken were quickly cooled to 4°C and held on ice until all the time points were taken. All the samples were then assayed for enzyme activity by normal procedures (37a), as given under "Experimental Procedure."

The KB and placental alkaline phosphatases have the same pH optimum (pH 11.0 in glycine buffer at 37°C). Both KB and placental alkaline phosphatase show 50% inhibition of enzyme activity at 5 x 10^-4 M phenylalanine or leucine. However, the KB phosphatase is considerably more stable to heating than is placental alkaline phosphatase (Fig. 10).

Comparison with HeLa Cells—Due to the claims that the KB cell line may have been contaminated with HeLa cells at some time (34), our KB cells were checked for HeLa cell "markers" (analyses performed by the Cell Culture Laboratory, University of California, Oakland). The KB cell line had the type A electrophoretic variant of glucose-6-phosphate dehydrogenase, generally considered an isozyme marker for HeLa cells, and had several HeLa "marker chromosomes" (Miller's markers No. 1, 3, and 4) as visualized by trypsin-Giemsa banding. However, Miller marker chromosome No. 2 was definitely absent.

The HeLa cell alkaline phosphatase differs from the KB phosphatase in several respects. In non-denaturing acrylamide gels without Triton X-100, HeLa phosphatase migrated as one band with a mobility (0.420) close to that of placental alkaline phosphatase (0.415) run on the same gel. KB phosphatase did not enter the gel unless Triton X-100 was present, and then always gave two enzyme bands with mobilities quite different from placental alkaline phosphatase.

HeLa cell membranes labeled with [32P]phosphate gave a single [32P]band (Fig. 9) corresponding to a molecular weight of 64,000 (the same as placental alkaline phosphatase). KB membranes always gave two [32P]-binding protein bands.

Neuraminidase treatment alters the mobility of the HeLa alkaline phosphatase enzyme activity on acrylamide gels without Na dodecyl-SO4, in the same direction and to the same extent that it alters placental alkaline phosphatase (Table III).

**DISCUSSION**

The existence of isoenzymes of alkaline phosphatase has been suggested in bacterial and mammalian systems. Multiple forms of alkaline phosphatase have been suggested for HeLa, KB, and other human cell lines. Previous claims of the existence of isoenzymes of alkaline phosphatase in the KB cell line (10) are based on equivocal data. This paper presents the first structural evidence that there are two different alkaline phosphatase subunit proteins in KB cells. The two KB subunit proteins are separated only by electrophoresis in polyacrylamide gels containing Na dodecyl-SO4. The evidence presented here suggests that both KB phosphatase subunits participate in the enzyme reaction, but it is not known how the subunits are associated in the active enzyme, i.e., whether they are homodimers or heterodimers.

The native KB phosphatase does not migrate into acrylamide gels without Na dodecyl-SO4, unless Triton X-100 is present. This may be due to aggregation of the proteins or to attached lipids or carbohydrates. In gels with the solubilizing agent Triton X-100, there are two KB phosphatase bands, both of which differ from placental alkaline phosphatase in electrophoretic mobility. Electrophoretic variants of placental alkaline phosphatase have not been demonstrated before in this gel system. We interpret the two KB enzyme bands as aggregates, rather than different isoenzymes, since each contains the same two nonidentical subunit proteins on Na dodecyl-SO4 acrylamide gels. The data presented strongly suggest the identity of the Mr = 64,000 KB phosphatase subunit to the monomer of placental alkaline phosphatase, and the close similarity of the Mr = 72,000 subunit. The Mr = 64,000 KB subunit and placental alkaline phosphatase monomer were identical in NH2-terminal amino acid sequence, molecular weight (mobility on Na dodecyl-SO4 gel), alteration of Na dodecyl-SO4 electrophoretic mobility by neuraminidase, and in binding [32P]phosphate. In addition to these data for the isolated Mr = 64,000 subunit, a number of experiments on the purified native KB enzyme strongly support its close similarity to placental alkaline phosphatase: the peptide map showing the close correlation of all ten KB peptide spots to placental alkaline phosphatase peptides, the identity of isoelectric point (net charge) of placental alkaline phosphatase and KB phosphatase, the immunologic identity of KB and placental phosphatase, [32P]phosphate binding, and reaction with specific antibody to placental alkaline phosphatase on affinity chromatography column, pH optimum, and inhibition of enzyme activity by phenylalanine and leucine.

The Mr = 72,000 KB subunit differs from the placental alkaline phosphatase monomer in at least one amino acid, in mobility on Na dodecyl-SO4 gel, and quantity of oxidizable carbohydrates (intensity of PAS staining). Against these differences, we have evidence of strong similarity of the Mr = 72,000 subunit to the placental alkaline phosphatase monomer: co-purification with the Mr = 64,000 subunit, two out of three NH2-terminal amino acids identical to placental alkaline phosphatase, [32P]phosphate binding, and reaction with specific antibody to placental alkaline phosphatase on affinity chromatography column. All the data of similarity of the native KB phosphatase to placental alkaline phosphatase would also support the strong similarity of the Mr = 72,000 KB subunit to the placental alkaline phosphatase monomer, since it comprises about one-half of the native KB enzyme.
Several differences were found between the KB alkaline phosphatase and placental alkaline phosphatase. Of these, the difficulty in solubilizing KB phosphatase for electrophoresis without Na dodecyl-SO₄, the different mobility on gels with Triton X-100, and different heat stability could all be due to differences in the carbohydrates attached to the protein chains. Attached carbohydrates have been shown to alter the enzymatic stability (35) and electrophoretic mobility (36) of other proteins. The \( M_r = 72,000 \) KB phosphatase subunit contains more oxidizable carbohydrate than does the \( M_r = 64,000 \) KB subunit. The amount or type of attached carbohydrates could account for the slower mobility on Na dodecyl-SO₄ gels of the \( M_r = 72,000 \) subunit. If the extra carbohydrates on the \( M_r = 72,000 \) subunit were not charged, the isoelectric point of native KB enzyme would be the same as if it were composed only of the \( M_r = 64,000 \) subunit.

The peptide map of KB phosphatase lacks one of the peptide spots present in the placental alkaline phosphatase map. However, the peptide which was missing in the map of KB phosphatase was a faint one in the placental alkaline phosphatase maps, and was not resolved in one-third of the placental alkaline phosphatase maps. A certain amount of variation in placement and resolution of peptide spots is common in peptide maps (37) and must be considered when interpreting the results.

If the peptide is truly missing in KB phosphatase and if it is very large, one would expect a difference in isoelectric point and possibly a lower molecular weight than placental alkaline phosphatase. These differences are not seen. Thus, if the peptide is missing, it is probably fairly small and does not contain many charged amino acids (or it has a net charge the same as the isoelectric point of the whole protein). Post-transcriptional cleavage of a portion of the protein molecule could have occurred. Post-transcriptional events are thought to be the cause of differences in the primary structure of isoenzymes of E. coli alkaline phosphatase, which is coded by a single gene (38, 39).

Given the limitations of the peptide mapping technique, and the possibility of post-transcriptional alteration of a small portion of the structure of the KB phosphatase, it is not possible to resolve definitively the genetic relation of the two KB phosphatase subunits and the subunit of placental alkaline phosphatase. However, our evidence strongly indicates the probable identity of the \( M_r = 64,000 \) KB subunit and the monomer of placental alkaline phosphatase. This would imply that the gene for placental alkaline phosphatase, which is normally repressed in all cells but the trophoblast, is derepressed in these tumor cells. The data presented also suggest that the \( M_r = 72,000 \) KB subunit is a very similar protein. Since both KB phosphatase subunits are produced by the same clone of KB cells, it is possible that there are multiple alleles of the gene for placental alkaline phosphatase in this tumor cell line.

On the basis of chromosomal and enzymatic similarities between KB and HeLa cells, it has been proposed that the KB cell line may have been contaminated with HeLa at some time (34). This possibility has not been ruled out by the present study. However, the fact that HeLa cells produce high levels of placental-like alkaline phosphatase is in itself significant, since normal endometrium does not produce any immunologically placental-type alkaline phosphatase. In addition, the production of placental-type alkaline phosphatase is a general phenomenon seen in human and rodent tumors and in at least one cell line other than HeLa and KB, i.e. ChaGo cells (33).

Furthermore, the enzyme and chromosome markers used to identify HeLa cells are not absolutely specific. A small percentage of the Caucasian population has the type A variant of glucose-6-phosphate dehydrogenase. Abnormal chromosomes can change with time in culture, and not all HeLa cell lines contain the same “marker” chromosomes. Similar abnormal chromosomes could be arrived at independently by different cell lines over decades in tissue culture.

In addition, there are differences between KB and HeLa cells, and between the KB and HeLa phosphatase. Our KB cells lacked at least one of the HeLa “marker chromosomes.” Alkaline phosphatase activity in HeLa cells is increased by prednisolone and hyperosmolality, while these treatments decrease the total alkaline phosphatase activity in KB cells (10). On polyacrylamide gels without Na dodecyl-SO₄, the native KB phosphatase gives two bands of enzyme activity, while HeLa cell extracts have only one phosphatase band. Finally, in \(^{32}P\) phosphate labeling experiments the two KB phosphatase subunits are equally labeled, whereas HeLa cell extracts show only one band of \(^{32}P\), at a molecular weight of 64,000.

Placental alkaline phosphatase is one of a number of fetal antigens which are expressed in transformed cells. In a significant number of cancer patients, three placental proteins—placental alkaline phosphatase, chorionic gonadotropin and placental lactogen—are found in the serum (40). If this phenomenon is indicative of gene derepression in the tumor cells, it is interesting to speculate why these fetal proteins are expressed in the neoplastic cells. First, it is possible that the gene for placental alkaline phosphatase is one of a number of genes that are randomly derepressed due to transformation. These genes may even be near an insertion site for a carcinogenic virus. Second, it may be that the genes for embryonic proteins are repressed (during adult life) in a qualitatively different way than the genes for other proteins, and thus they may be derepressed more frequently after neoplastic transformation. Lastly, it is possible that placental alkaline phosphatase may be useful to the tumor cells. Placental alkaline phosphatase is normally located on the plasma membrane of the trophoblast, a tissue that is in some ways similar to tumor cells (high metabolism, invasive behavior). It may be that placental alkaline phosphatase plays some role in the altered cell surface properties of tumor cells.

Acknowledgments—The authors are very grateful to Drs. David Sedwick, Richard Ludueña, and H. Garrett Wada and Mr. Robert Carlson for criticism of this manuscript. We also wish to thank Ms. Kirsten Badger for stimulating discussions and for technical assistance with the affinity chromatography.

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Characterization of KB cell alkaline phosphatase. Evidence of similarity to placental alkaline phosphatase.
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