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Structural analysis of human adult and fetal alkaline phosphatases by cyanogen bromide peptide mapping

(immunoaffinity chromatography/radioiodination/differential glycosylation/developmental regulation)

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ABSTRACT **The adult and fetal forms of human intestinal alkaline phosphatase (ALPase; orthophosphoric-monoester phosphohydrolase, EC 3.1.3.1) are indistinguishable by a variety of analytical procedures. However, they differ electrophoretically and can be differentiated by binding studies with monoclonal antibodies. In this report, these two enzymes along with placental and liver ALPases are compared by the technique of CNBr peptide mapping, and the role of carbohydrate in generating these patterns is investigated. NaDodSO$_4$/PAGE of CNBr digests of radiolabeled ALPases from fetal and adult intestine shows that these two isozymes share five of seven common-sized CNBr fragments. Placental ALPase shares only one common-sized fragment with either intestinal enzyme. Liver ALPase has no CNBr fragments in common with any of the others. These data indicate that fetal intestinal ALPase is not a heterodimer of one subunit each of intestinal ALPase and placental ALPase as has been postulated. CNBr digests of neuraminidase-treated enzymes reveal a change of mobility of only one CNBr band in each of fetal intestinal, placental, and liver ALPases, indicating the presence of sialic acid residues in these fragments. Periodic acid/Schiff reagent staining (specific for carbohydrate) of CNBr digests of fetal and adult intestinal ALPases reacts with only one band in each enzyme, which is the same band from the fetal enzyme shown to contain sialic acid. However, fetal and adult intestinal ALPases each contain at least one CNBr fragment of unique size that is apparently nonglycosylated.**

The human alkaline phophatases [ALPases; orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1] are a multigene family encoded by at least three gene loci (1–4). They are membrane-bound glycoproteins produced in different forms and at various levels in a number of tissues (reviewed in ref. 5). Liver, bone, and kidney ALPase are coded for by at least one gene. Placental ALPase is encoded by a second locus and is produced in large quantities only in the placenta under the control of the fetal genome (6). A third type of ALPase is found in intestinal mucosa and is the product of at least one further locus.

Two forms of intestinal ALPase have been recognized, adult and fetal. ALPase from adult and fetal intestinal mucosa show identical thermostabilities and sensitivities to a variety of enzymatic inhibitors and also react identically with polyclonal rabbit antisera raised to fetal intestinal ALPase and to placental ALPase (7–10). However, adult and fetal intestinal ALPases can be distinguished electrophoretically, due at least in part to the presence of sialic acid on the fetal form but not on the adult form of the enzyme (7). But, even after the removal of sialic acid, the enzymes do not comigrate. The intestinal isozymes also show different subunit molecular weights on NaDodSO$_4$/PAGE (11). Two-dimensional tryptic digest peptide maps of adult and fetal intestinal ALPases have been reported to be similar but not identical (10). In the literature, there is disagreement over the amino-terminal amino acid of the fetal enzyme. One group reports that fetal intestinal ALPase has the same amino-terminal amino acid as in the adult enzyme (10), while another finds that they are different (9). Finally, Behrens *et al.* (12) have recently suggested that fetal intestinal ALPase is a heterodimer composed of one subunit of placental ALPase and one of adult intestinal ALPase.

In this paper, the technique of CNBr peptide mapping is used to compare purified adult intestinal, fetal intestinal, placental, and liver ALPases. In addition, those CNBr fragments from adult and fetal intestinal ALPases that stain with the carbohydrate-specific periodic acid/Schiff reagent (PAS) and also those that contain sialic acid as shown by neuraminidase treatment are identified. NaDodSO$_4$/PAGE of CNBr digests of the ALPases resolves seven fragments each for adult and fetal intestinal ALPases, five of similar size and two of dissimilar size. Placental ALPase shares one common-sized fragment with both intestinal enzymes, while liver ALPase has no CNBr fragments in common with any of the others. PAS staining of CNBr digests of both intestinal enzymes identifies one of the two fragments that distinguishes adult and fetal ALPases as a glycopeptide. The same fragment from the fetal enzyme changes mobility when the enzyme is desialated prior to CNBr digestion.

These results indicate that fetal intestinal ALPase cannot be composed of one subunit of adult intestinal ALPase and one of placental ALPase. Furthermore, in conjunction with monoclonal antibody studies (see *Discussion*), the data suggest that adult and fetal forms of intestinal ALPase may be structurally distinct proteins.

MATERIALS AND METHODS

ALPase Assays. ALPase activity was assayed as described (1).

Neuraminidase Treatment. Samples were treated with neuraminidase as described (7).

Monoclonal Antibodies to ALPases. ALP$_p$/Sp2/2, a monoclonal antibody to placental ALPase, has been described (13). Ascites fluid, rich in anti-ALPase monoclonal antibodies, was produced by intraperitoneal injection of hybridoma cells with pristane-primed mice.

Purification of Placental ALPase by Immunoaffinity Chromatography. CNBr-activated Sepharose 4B (1.5 g, Pharmacia) was swollen in 1.0 mM HCl for 15 min at room temperature with gentle mixing. The gel was washed with 0.1 M $NaHCO_3$, pH 8.3/0.5 M NaCl (bicarbonate buffer) and mixed overnight at 4°C with 3.0 ml of ALP$_p$/Sp2/2 ascites fluid. The gel was then washed with bicarbonate buffer, and all remaining active groups were blocked by resuspending

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Abbreviations: ALPase, alkaline phosphatase; PAS, periodic acid/Schiff reagent.

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the beads in 0.2 M glycine (pH 8.0) for 2 hr at room temperature. Excess, noncoupled protein was removed by washing the beads with three cycles of bicarbonate buffer alternating with 0.1 M sodium acetate, pH 4.0/0.5 M NaCl, followed by a final washing with bicarbonate buffer. The beads were then packed into a 0.9 × 15 cm column and equilibrated with 0.05 M Tris·HCl, pH 7.4/0.1 M NaCl/5.0 mM $MgCl_2$. Type 1 placental extract, prepared with a butanol extraction procedures as described (14), was loaded onto the column and washed extensively with 0.05 M Tris·HCl/5.0 mM $MgCl_2$, pH 7.4, buffer. Placental ALPase was eluted with 1 M NH_4OH. All fractions were immediately neutralized by the addition of 1 M Tris·HCl (pH 7.0). Purified enzyme was concentrated by ultrafiltration, desalted on a Sephadex G-25 column (Pharmacia), and stored at −20°C until use. Purity was determined by PAGE under denaturing and nondenaturing conditions as described below.

Purification of Adult and Fetal Intestinal ALPases and Liver ALPase. Purification of these enzymes by monoclonal antibody immunoaffinity chromatography has been described (11, 15).

Radioiodination of ALPases. Purified ALPases from placenta, adult intestine, meconium (rich in fetal intestinal ALPase), and liver were used at a concentration of 1.0 mg/ml. Purified protein (50 μg) was mixed with 30 μl of chloramine-T (0.5 mg/ml; Sigma) and immediately was added to a tube containing 1.0 mCi of $Na^{125}I$ (Amersham; 1 Ci = 37 GBq). After 30 sec, the reaction was stopped by the addition of a saturated tyrosine solution in 0.5 M phosphate buffer (pH 7.4), and radiolabeled protein was separated from free ^{125}I by gel filtration on a Sephadex G-25 (medium) column (Pharmacia).

CNBr Cleavage of ALPases. An aliquot of radiolabeled protein containing about 10^8 cpm was diluted into 1 ml of 7 M guanidine·HCl/0.5 M Tris·HCl/2 mM EDTA, pH 8.2. Nonradioactive ovalbumin (Sigma) was added to bring the protein concentration to 2.0 mg/ml. 2-Mercaptoethanol was then added to give a final concentration of 0.03 M, and the mixture was incubated at 37°C for 2 hr. Iodoacetic acid was added to a final concentration of 0.035 M to alkylate exposed sulfhydryl groups. After a 15-min incubation at room temperature, the protein was desalted on a G-25 Sephadex column previously equilibrated with 0.5 M NH_4OH. Samples were then lyophilized and resuspended in 70% (vol/vol) formic acid. About 2 mg of solid CNBr (Eastman) was added, and the reaction allowed to proceed at room temperature for 24 hr. The sample was then diluted into about 20 ml of distilled H_2O and lyophilized. Unlabeled enzyme (1.0 mg) was digested in the same manner except that ovalbumin carrier was not added.

Polyacrylamide Gel Electrophoresis. After denaturation and reduction by boiling in 0.5% $NaDodSO_4$/2% (vol/vol) 2-mercaptoethanol, the CNBr-digested ALPases were subjected to electrophoresis on 20% polyacrylamide gel by the method of Laemmli (16).

Identification of Glycopeptides of Unlabeled ALPases. A modified PAP technique was used to identify the CNBr fragments from fetal and adult intestinal ALPases that contained carbohydrate (17). Purified enzyme (1.0 mg) was digested with CNBr as above and boiled in $NaDodSO_4$ sample buffer, and about 250 μg of the sample was subjected to electrophoresis on a $NaDodSO_4$/20% polyacrylamide gel. The gel was then soaked for 90 min in 40% (vol/vol) methanol/7% (vol/vol) acetic acid at room temperature with two changes and incubated for 60 min in 1% periodic acid/7% (vol/vol) acetic acid at 4°C with two changes. The gel was then treated with Schiff reagent (Sigma) for 60 min at 4°C, and the banding pattern was traced on a piece of Mylar plastic film (Imperial Chemical Industries, Macclesfield, England) overlayed on the gel. The gel was washed for 90 min with 1% sodium bisulfite in 0.1 M HCl at room temperature and then stained for protein with Coomassie blue.

RESULTS

Purification and Radioiodination of ALPases. Monoclonal antibody immunoaffinity chromatography was used to obtain highly purified (>99% as estimated by $NaDodSO_4$/PAGE) samples of placental, liver, fetal intestinal, and adult intestinal ALPases in high yields. The proteins were analyzed for purity by $NaDodSO_4$/PAGE both before and after radioiodination. Fig. 1 shows the purified, radiolabeled enzymes.

CNBr Peptide Mapping of the Radiolabeled ALPases. To investigate the structural relationship of the various ALPases, we used the technique of CNBr peptide mapping. Fig. 2 shows one such CNBr digest of the four radioiodinated isozymes before and after desialation. The accompanying diagram represents a composite of the results obtained from several independent experiments. The CNBr peptides resolved by this system are numbered. The unnumbered, higher molecular weight fragments were shown by appropriate controls to represent undigested protein or peptides generated by treatment of the protein with formic acid alone. Seven CNBr fragments were reproducibly resolved from adult and fetal intestinal ALPase, five from placental ALPase, and four from liver ALPase. The sums of the molecular weights of the CNBr fragments resolved for adult intestinal, fetal intestinal, and placental ALPases are roughly equivalent to their subunit molecular weights. This sum is considerably lower than expected for liver ALPase; however, band L3 appears to be heterogeneous and probably represents more than one peptide. The intestinal ALPases and placental ALPase showed no common-sized peptide fragments with liver ALPase, whereas placental ALPase shared one common-sized fragment with both fetal and adult intestinal ALPases (peptides F6, A6, and P5). Of the seven peptide fragments resolved from adult and fetal intestinal ALPases, five were similar in size, while two were different.

For each of the isozymes known to contain sialic acid, the mobility of only one peptide fragment was affected by neuraminidase treatment: F_4, P_4, and L_3 as labeled in Fig. 2. No mobility changes were seen after neuraminidase treatment of adult intestinal ALPase, as expected. The small change in mobility of band F_4 after neuraminidase treatment was reproducible under a variety of electrophoretic conditions for CNBr digestions from a number of different experiments and could be exaggerated by a longer electrophoretic run.

PAS Staining of Adult and Fetal Intestinal ALPase CNBr Peptides. To identify the glycopeptides from CNBr digests of adult and fetal intestinal ALPases, irrespective of sialic acid residues, we used a modified PAS technique (17). This required a great deal more protein than was needed to visualize the radioiodinated proteins and proved to be about the same

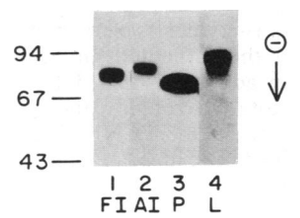

FIG. 1. Autoradiograph of purified, radiolabeled ALPases after $NaDodSO_4$/PAGE using a 7.5% gel. The relative positions of molecular mass standards (in kilodaltons) are indicated. Lanes: 1, fetal intestinal (FI) ALPase; 2, adult intestinal (AI) ALPase; 3, placental (P) ALPase; 4, liver (L) ALPase. Lanes 1 and 4 were exposed for 15 min. Lanes 2 and 3 were exposed for 1 hr. Electrophoresis was for 19 hr at 8-mA constant current.

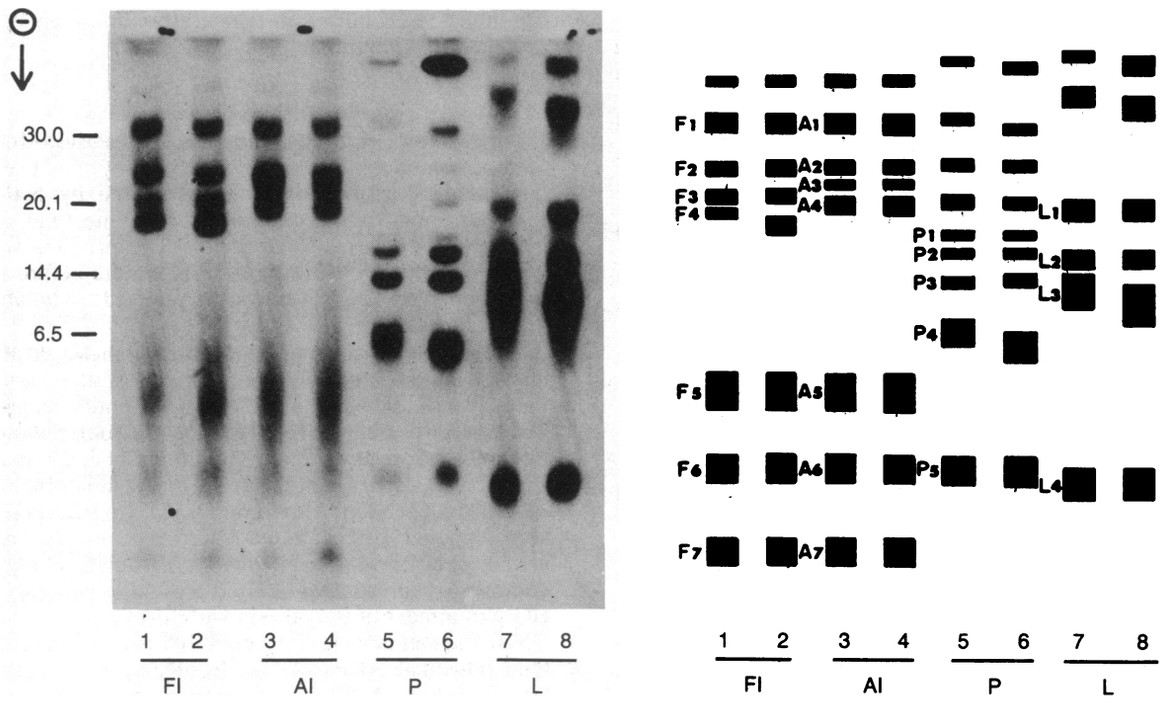

FIG. 2. (*Left*) CNBr peptide map of purified, radiolabeled ALPases before and after neuraminidase treatment. Only true CNBr peptides are numbered. (*Right*) Drawing representing a composite of this and other experiments. Lanes: 1, 3, 5 and 7, CNBr digests of ALPases prior to neuraminidase treatment; 2, 4, 6 and 8, CNBr digests of neuraminidase-treated ALPases. The autoradiograph was exposed for 19 hr. Electrophoresis was for 7 hr at 35-mA constant current using a $NaDodSO_4$/20% polyacrylamide gel. Molecular weight markers (in kilodaltons) are carbonic anhydrase, soybean trypsin inhibitor, α-lactalbumin, and aprotinin. ALPases: FI, fetal intestinal; AI, adult intestinal; P, placental; L, liver.

quantity as was necessary to stain the fragments with a general protein stain such as Coomassie blue. The sensitivity of the stain for purified adult and fetal intestinal ALPases was first tested on undigested enzyme after $NaDodSO_4$/PAGE using 10% gels. Both enzymes stained approximately equally and about as strongly as a control of purified human transferrin (carbohydrate content of 11.4 g/100 g of protein), with a limit of sensitivity of about 10 μg of enzyme. Correlation of the CNBr patterns obtained with the PAS and Coomassie staining with that obtained by using the iodinated enzymes was accomplished by running all samples on the same gel. The findings are shown diagrammatically in Fig. 3 and can be summarized as follows: for both intestinal enzymes, the Coomassie blue pattern was the same as the ^{125}I pattern except that Coomassie blue did not stain the two lowest molecular weight bands (F_6, F_7, A_6, and A_7). Only one of the CNBr peptides from each of the two enzymes stained darkly with the PAS technique, corresponding to bands F_4 and A_4.

DISCUSSION

Amino acid composition data (and specifically methionine content) for these isozymes is very limited. However, because the sum of the estimated molecular weights of the CNBr peptides for adult intestinal, fetal intestinal, and placental ALPases total to roughly their respective subunit sizes, it would seem that most CNBr fragments for these enzymes were resolved. The maps of liver, placental, and adult intestinal ALPases are all different, which is consistent with their assignment to separate gene loci (1–4). Liver ALPase shares no common-sized peptides with placental ALPase or either of the intestinal ALPases. Placental ALPase shares one common-sized CNBr fragment with both intestinal ALPases. Finally, radioiodinated fetal intestinal ALPase shares five of seven common-sized fragments with adult intestinal ALPase and only one common-sized fragment with placental ALPase. The fetal intestinal ALPase pattern clearly cannot be explained by an equimolar mixing of adult intestinal ALPase and placental ALPase as would be the case if, as suggested by Behrens *et al.* (12), fetal intestinal ALPase is a heterodimer composed of one subunit each of adult intestinal and placental ALPases.

There are several possible explanations for the observed differences in the peptide maps of fetal and adult intestinal

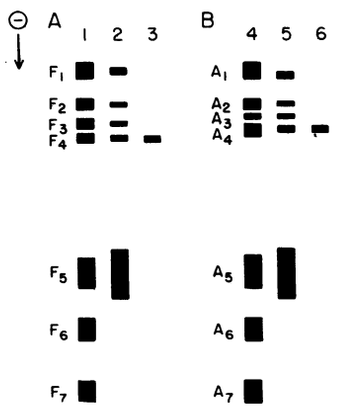

FIG. 3. Comparison of CNBr maps of radiolabeled (lanes 1 and 4) fetal (*A*) and adult (*B*) intestinal ALPases and of the nonradiolabeled intestinal isozymes stained with Coomassie blue (lanes 2 and 5) and PAS (lanes 3 and 6). Unlabeled enzyme was digested with CNBr and subjected to electrophoresis on a $NaDodSO_4$/20% polyacrylamide gel alongside a CNBr digest of a radiolabeled sample, at 35-mA constant current for 7 hr. The labeled sample was sliced off and processed for autoradiography. The remainder of the gel was stained with the PAS technique and then with Coomassie blue. Undigested and formic acid-treated ALPases (radiolabeled and nonradiolabeled) were also run as controls. Relative mobilities of all bands visualized with the three methods were calculated. Shown is a composite drawing of the banding pattern of true CNBr fragments from fetal (*A*) and adult (*B*) intestinal ALPases.

ALPases. One possibility is that the proteins may be encoded by the same gene and that the differences in CNBr maps could be due to post-translational changes involving the addition of carbohydrate. To examine the role of carbohydrate in generating the differences in the CNBr maps of the ALPases, the CNBr fragments were analyzed for carbohydrate content by a modified PAS staining technique for carbohydrate (17). In addition, those fragments from fetal intestinal, placental, and liver ALPases containing sialic acid residues—an additional marker of peptide glycosylation—were identified. The PAS technique is an oxidation–reduction reaction which, under appropriate conditions, is specific for hydroxyl groups on adjacent carbon atoms. It has been applied widely in cytochemistry as a general carbohydrate stain. For staining to be specific after $NaDodSO_4$/PAGE (as defined with known glycoprotein and nonglycoprotein standards), the incubation conditions described in *Materials and Methods* had to be followed rigidly. Longer reaction times or incubation at warmer temperatures resulted in the nonspecific staining of all proteins in the gel. Under the described conditions, undigested purified adult and fetal intestinal ALPases stained equally well, and the reaction could be stopped before nonspecific staining of undigested and CNBr-digested control proteins occurred.

There is a good correlation between the ^{125}I pattern and Coomassie blue-stained pattern of CNBr digests of fetal and adult intestinal ALPases. Only the two lowest molecular weight ^{125}I-labeled bands (F_6, F_7, and A_6, A_7) were not visible with the Coomassie stain. This is most likely due to their small size (<3000 daltons). For fetal intestinal ALPase, the only ^{125}I-labeled band shown to contain a sialic acid residue (F_4) is also the only CNBr fragment found to stain with PAS. Similarly, only one CNBr fragment from adult intestinal ALPase stains with PAS (corresponding to the ^{125}I-labeled band A_4). This represents only one of the two CNBr fragments from both intestinal ALPases that differ in their apparent molecular size.

These data indicate that fetal intestinal ALPase does not simply represent a heterodimer composed of one subunit each of adult intestinal and placental ALPases. Furthermore, although the two intestinal isozymes yield very similar CNBr peptide maps, they are nonetheless distinct. Since one of the peptides that differs between the two isozymes does not contain detectable carbohydrate, this difference does not arise at the level of post-translational glycosylation. Additional experiments are necessary to determine specifically at what level these differences arise.

In other studies, 11 monoclonal antibodies that react with both intestinal ALPases have been produced in this laboratory: 6 were raised to fetal intestinal ALPase (18), 1 to placental ALPase (19), and 4 to an intestinal-like ALPase found in the human cell line D98/AH-2 (20). Of these, 9 can discriminate between adult and fetal intestinal ALPases in binding studies. One of these antibodies (raised to fetal intestinal ALPase and cross-reacting with placental ALPase) appears to recognize structural differences at the protein level since it differentiates the product of one of the common alleles at the polymorphic placental ALPase locus from the others (18). Thus, it is possible that the observed differences in CNBr peptide maps between adult and fetal intestinal ALPases may be at the protein level. If so, the two proteins may be encoded by separate gene loci, or alternatively, may arise from differential processing at the RNA or protein level of the product of a single gene.

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