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Cloning and sequencing of human intestinal alkaline phosphatase cDNA

(isoenzyme)

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ABSTRACT **Partial protein sequence data obtained on intestinal alkaline phosphatase indicated a high degree of homology with the reported sequence of the placental isoenzyme. Accordingly, placental alkaline phosphatase cDNA was cloned and used as a probe to clone intestinal alkaline phosphatase cDNA. The latter is somewhat larger (3.1 kilobases) than the cDNA for the placental isozyme (2.8 kilobases). Although the 3′ untranslated regions are quite different, there is almost 90% homology in the translated regions of the two isozymes. There are, however, significant differences at their amino and carboxyl termini and a substitution of an alanine in intestinal alkaline phosphatase for a glycine in the active site of the placental isozyme.**

In animals, alkaline phosphatase activity [orthophosphoric-monoester phosphohydrolases (alkaline optimum), EC 3.1.3.1] is found essentially in all tissues. In almost all mammals, the most abundant isoenzyme of alkaline phosphatase is the one found in liver, kidney, bone, and most other tissues and called tissue-unspecific alkaline phosphatase (AP). A second isozyme is found in greatest abundance in the intestine (IAP) of all mammals. In humans and higher primates, a third isozyme appears in term placenta (PLAP). Although PLAP was originally considered to be uniquely present in placenta and in tumors of ectopic origin (1), it has also been found in appreciable quantity in normal human liver and intestine (2).

Although the physiological function(s) of the alkaline phosphatases is still not known, considerable information has begun to appear concerning their molecular structure. Amino-terminal sequencing of all three human isozymes has recently been reported (3). In that study, greater than 90% homology was shown between PLAP and IAP at their amino termini (39 residues). The cDNA of variants of one of the isozymes, PLAP, have now been cloned and sequenced in several laboratories (4–7). Cloning of the other isozymes is necessary to understand the relationships among the isozymes, to provide tools for elucidating the function and regulation of the alkaline phosphatases, and to determine their mode of attachment to plasma membranes. In this report we present the cloning and sequencing of IAP cDNA and make comparisons with PLAP.

METHODS AND MATERIALS

Reagents. The λgt10 arms and the Gigapack kit were obtained from Stratagene (San Diego, CA). [α-^{32}P]dATP (5000 Ci/mmol; 1 Ci = 37 GBq), [α-^{32}P]dCTP (6000 Ci/mmol), and [α-[^{35}S]thio]dATP (600 Ci/mmol) were obtained from Amersham (Arlington Heights, IL). The various DNA- and RNA-modifying enzymes and restriction endonucleases were obtained from Amersham, Bethesda Research Laboratories, Boehringer Mannheim, International Biotechnologies (New Haven, CT), New England Biolabs, and Pharmacia.

DNA Sequencing. cDNA inserts to be sequenced were subcloned into phages M13mp18 and M13mp19. The DNA sequences of both strands were determined by the dideoxynucleotide chain-termination technique (8) after the generation of overlapping exonuclease III deletions by the method of Henikoff (9).

Cloning PLAP cDNA for Use as a Probe to Screen a Human Intestinal cDNA Library. A λgt10 library consisting of about 500,000 independent recombinant plaques was constructed according to the method of Huynh *et al.* (10), using cDNA derived from placental polyadenylylated mRNA. The latter was isolated from a single term placenta obtained from Mountainside Hospital (Montclair, NJ). The cDNA was prepared *in vitro* according to the method of Gubler and Hoffmann (11), and about 100 ng were used for construction of a library. Screening was carried out by the tetramethylammonium chloride procedure (12) using two oligodeoxyribonucleotides (24 and 26 bases each) encompassing nucleotide regions 600–624 and 1495–1521 of the published sequence of PLAP (4). Four independent clones were obtained, two of which contained a full-length insert for PLAP. Seventy-five percent of one of the inserts was sequenced and proved to be identical to the one reported by Millan (5). This full-length PLAP insert was subcloned into pBR322 according to standard procedures and nick-translated (13) for use as a probe to screen the intestinal cDNA library.

Preparation and Screening of a λgt10 Intestinal cDNA Library. Human small intestine was obtained from the Sloan–Kettering Cancer Research Center. The specimen, obtained from an adult subject who had died from tumors unrelated to intestinal neoplasia, showed no evidence of malignancy according to the autopsy report. The intestine was stored at −70°C until use. RNA was extracted by the guanidinium isothiocyanate procedure followed by CsCl centrifugation (13). Total polyadenylylated RNA was selected by oligo(dT)-cellulose chromatography (13). Synthesis of the cDNA was carried out using the method of Gubler and Hoffmann (11). Approximately 200 ng of the cDNA were used to construct a λgt10 library consisting of about 750,000 independent recombinant plaques. The library was screened with the PLAP probe prepared as described above. Hybridization was carried out at 53°C in a solution consisting of 5× SSC, 50 mM Na_2HPO_4/NaH_2PO_4 (pH 6.5), 5× Denhardt's solution, 0.1% $NaDodSO_4$, and heat-denatured salmon sperm DNA (20 μg/ml), *Escherichia coli* DNA (20 μg/ml), and poly(adenylic acid) (1 μg/ml). (1× SSC is 0.15 M NaCl/0.015

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Abbreviations: PLAP, placental alkaline phosphatase; IAP, intestinal alkaline phosphatase.

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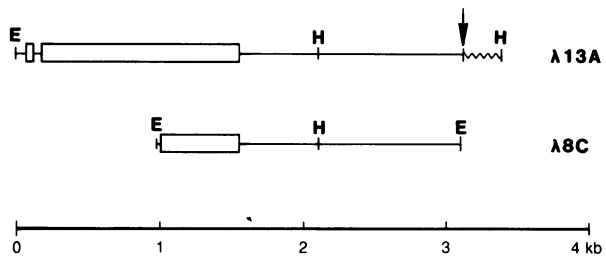

FIG. 1. Schematic representation of human IAP cDNA clones λ13A and λ8C. Open boxes represent translated regions. Solid lines represent untranslated regions. The zigzag line represents λgt10 sequence. The arrow indicates the position where the synthetic linker *Eco*RI recognition site has been lost. Restriction sites: E, *Eco*RI; H, *Hin*dIII.

M sodium citrate, pH 7; 1× Denhardt's solution is 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin.) The filters were washed under nonstringent conditions in 2× SSC/0.1% $NaDodSO_4$ at 53°C.

RESULTS AND DISCUSSION

Sequencing of Tryptic Peptides. Human IAP (700 pmol) was purified as described (3). The reduced and carboxymethylated enzyme was treated with trypsin and the tryptic peptides were separated by HPLC on a C_{18} column using a propanol/pyridine acetate buffer system (14). Approximately 30 peptide peaks appeared. Eleven of the resolved peptides were sequenced on an Applied Biosystems sequencer (Foster City, CA). Three of the tryptic peptides coincided with the amino-terminal sequence that was reported previously (3). Eight peptides were obtained that provided additional sequence information. These are shown in Fig. 2.

Cloning of IAP cDNA. Protein sequence data from the amino-terminal regions and from peptides obtained by trypsin cleavage of IAP (Fig. 2) indicated a high degree of homology with PLAP (4) and suggested that the homology extended along the entire protein chain. This prompted us to first clone PLAP cDNA and subsequently use it as a probe to screen an intestinal cDNA library. Two positive plaques were obtained. After two rounds of plaque purification the positive plaques were found to contain two related inserts, clone λ8C [≈2.1 kilobases (kb)] and clone λ13A (≈3.4 kb) (Fig. 1). The insert from clone λ13A could not be released from the phage by *Eco*RI digestion. This problem was circumvented by taking advantage of a nearby *Hin*dIII site in the long arm of λgt10. Digestion of λ13A with *Eco*RI and *Hin*dIII yielded two fragments of approximately 1.3 and 2.1 kb, demonstrating an internal *Hin*dIII site.

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                -19                                  -9
CCCAAGAC ATG CAG GGG CCC TGG GTG CTG CTG CTG CTG GGC CTG AGG CTA CAG      53
         Met Gln Gly Pro Trp Val Leu Leu Leu Leu Gly Leu Arg Leu Gln

                1
CTC TCC CTG GGC GTC ATC CCAGGTAATGAGGCTCCCCAAGCTGTTCCACACACAGGGCACCC     115
Leu Ser Leu Gly Val Ile

CCTCAGCCAGGCTGACCTGATCTCTACTCTCCCCCTGG CCA GCT GAG GAG GAG AAC CCG       174
                                       Pro Ala Glu Glu Glu Asn Pro

10                                  20
GCC TTC TGG AAC CGC CAG GCA GCT GAG GCC CTG GAT GCT GCC AAG AAG CTG      225
Ala Phe Trp Asn Arg Gln Ala Ala Glu Ala Leu Asp Ala Ala Lys Lys Leu

          30                                  40
CAG CCC ATC CAG AAG GTC GCC AAG AAC CTC ATC CTC TTC CTG GGC GAT GGG      276
Gln Pro Ile Gln Lys Val Ala Lys Asn Leu Ile Leu Phe Leu Gly Asp Gly

                    50                                  60
TTG GGG GTG CCC ACG GTG ACA GCC ACC AGG ATC CTA AAG GGG CAG AAG AAT      327
Leu Gly Val Pro Thr Val Thr Ala Thr Arg Ile Leu Lys Gly Gln Lys Asn

                          70
GGC AAA CTG GGG CCT GAG ACG CCC CTG GCC ATG GAC CGC TTC CCA TAC CTG      378
Gly Lys Leu Gly Pro Glu Thr Pro Leu Ala Met Asp Arg Phe Pro Tyr Leu

          80                                  90
GCT CTG TCC AAG ACA TAC AAT GTG GAC AGA CAG GTG CCA GAC AGC GCA GCC      429
Ala Leu Ser Lys Thr Tyr Asn Val Asp Arg Gln Val Pro Asp Ser Ala Ala

                    100                                 110
ACA GCC ACG GCC TAC CTG TGC GGG GTC AAG GCC AAC TTC CAG ACC ATC GGC      480
Thr Ala Thr Ala Tyr Leu Cys Gly Val Lys Ala Asn Phe Gln Thr Ile Gly

                          120       *
TTG AGT GCA GCC GCC CGC TTT AAC CAG TGC AAC ACG ACA CGC GGC AAT GAG      531
Leu Ser Ala Ala Ala Arg Phe Asn Gln Cys Asn Thr Thr Arg Gly Asn Glu

    130                                 140
GTC ATC TCC GTG ATG AAC CGG GCC AAG CAA GCA GGA AAG TCA GTA GGA GTG      582
Val Ile Ser Val Met Asn Arg Ala Lys Gln Ala Gly Lys Ser Val Gly Val

          150                                 160
GTG ACC ACC ACA CGG GTG CAG CAC GCC TCG CCA GCC GGC ACC TAC GCA CAC      633
Val Thr Thr Thr Arg Val Gln His Ala Ser Pro Ala Gly Thr Tyr Ala His

                    170
ACA GTG AAC CGC AAC TGG TAC TCA GAT GCT GAC ATG CCT GCC TCA GCC CGC      684
Thr Val Asn Arg Asn Trp Tyr Ser Asp Ala Asp Met Pro Ala Ser Ala Arg

180                                 190
CAG GAG GGG TGC CAG GAC ATC GCC ACT CAG CTC ATC TCC AAC ATG GAC ATT      735
Gln Glu Gly Cys Gln Asp Ile Ala Thr Gln Leu Ile Ser Asn Met Asp Ile

          200                                 210
GAC GTG ATC CTT GGC GGA GGC CGC AAG TAC ATG TTT CCC ATG GGG ACC CCA      786
Asp Val Ile Leu Gly Gly Gly Arg Lys Tyr Met Phe Pro Met Gly Thr Pro

                    220                                 230
GAC CCT GAG TAC CCA GCT GAT GCC AGC CAG AAT GGA ATC AGG CTG GAC GGG      837
Asp Pro Glu Tyr Pro Ala Asp Ala Ser Gln Asn Gly Ile Arg Leu Asp Gly

                          240
AAG AAC CTG GTG CAG GAA TGG CTG GCA AAG CAC CAG GGT GCC TGG TAT GTG      888
Lys Asn Leu Val Gln Glu Trp Leu Ala Lys His Gln Gly Ala Trp Tyr Val

    *  250                                 260
TGG AAC CGC ACT GAG CTC ATG CAG GCG TCC CTG GAC CAG TCT GTG ACC CAT      939
Trp Asn Arg Thr Glu Leu Met Gln Ala Ser Leu Asp Gln Ser Val Thr His

                          270                                 280
CTC ATG GGC CTC TTT GAG CCC GGA GAC ACG AAA TAT GAG ATC CAC CGA GAC      990
Leu Met Gly Leu Phe Glu Pro Gly Asp Thr Lys Tyr Glu Ile His Arg Asp

                          290
CCC ACA CTG GAC CCC TCC CTG ATG GAG ATG ACA GAG GCT GCC CTG CGC CTG     1041
Pro Thr Leu Asp Pro Ser Leu Met Glu Met Thr Glu Ala Ala Leu Arg Leu

          300                                 310
CTG AGC AGG AAC CCC CGC GGC TTC TAC CTC TTT GTG GAG GGC GGC CGC ATC     1092
Leu Ser Arg Asn Pro Arg Gly Phe Tyr Leu Phe Val Glu Gly Gly Arg Ile

                    320                                 330
GAC CAT GGT CAT CAT GAG GGT GTG GCT TAC CAG GCA CTC ACT GAG GCG GTC     1143
Asp His Gly His His Glu Gly Val Ala Tyr Gln Ala Leu Thr Glu Ala Val

                          340
ATG TTC GAC GAC GCC ATT GAG AGG GCG GGC CAG CTC ACC AGC GAG GAG GAC     1194
Met Phe Asp Asp Ala Ile Glu Arg Ala Gly Gln Leu Thr Ser Glu Glu Asp

350                                 360
ACG CTG ACC CTC GTC ACC GCT GAC CAC TCC CAT GTC TTC TCC TTT GGT GGC     1245
Thr Leu Thr Leu Val Thr Ala Asp His Ser His Val Phe Ser Phe Gly Gly

          370                                 380
TAC ACC TTG CGA GGG AGC TCC ATC TTC GGG TTG GCC CCC AGC AAG GCT CAG     1296
Tyr Thr Leu Arg Gly Ser Ser Ile Phe Gly Leu Ala Pro Ser Lys Ala Gln

                    390                                 400
GAC AGC AAA GCC TAC ACG TCC ACT CTG TAC GGC AAT GGC CCG GGC TAC GTG     1347
Asp Ser Lys Ala Tyr Thr Ser Thr Leu Tyr Gly Asn Gly Pro Gly Tyr Val

                          410
TTC AAC TCA GGC GTG CGA CCA GAC GTG AAT GAG AGC GAG AGC GGG AGC CCC     1398
Phe Asn Ser Gly Val Arg Pro Asp Val Asn Glu Ser Glu Ser Gly Ser Pro

          420                                 430
GAT TAC CAG CAG CAG GCG GCG GTG CCC CTG TCG TCC GAG ACC CAC GGA GGC     1449
Asp Tyr Gln Gln Gln Ala Ala Val Pro Leu Ser Ser Glu Thr His Gly Gly

                    440                                 450
GAA GAC GTG GCG GTG TTT GCG CGC GGC CCG CAG GCG CAC CTG GTG CAT GGT     1500
Glu Asp Val Ala Val Phe Ala Arg Gly Pro Gln Ala His Leu Val His Gly

                          460
GTG CAG GAG CAG AGC TTC GTA GCG CAT GTC ATG GCC TTC GCT GCC TGT CTG     1551
Val Gln Glu Gln Ser Phe Val Ala His Val Met Ala Phe Ala Ala Cys Leu

    470                                 480
GAG CCC TAC ACG GCC TGC GAC CTG GCG CCT CCC GCC TGC ACC ACC GAC GCC     1602
Glu Pro Tyr Thr Ala Cys Asp Leu Ala Pro Pro Ala Cys Thr Thr Asp Ala

                    490                                 500
GCG CAC CCA GTT GCC GCG TCG CTG CCA CTG CTG GCC GGG ACC CTG CTG CTG     1653
Ala His Pro Val Ala Ala Ser Leu Pro Leu Leu Ala Gly Thr Leu Leu Leu

                          509
CTG GGG GCG TCC GCT GCT CCC TGA GTGCCCCACTCCGGAGTTATCCTGCTCCCCACCTC     1712
Leu Gly Ala Ser Ala Ala Pro ---

GGGCGTCCTGCCCTGTTCCCGGTCCTGAGCCGCCATTCCAGCGAACACACACAGGTGTCCTGCCGTT     1779
GGACCTTCACCTCCTAGAGATAAACCAGCCTCAG-(          )-TACATTTACAAAGGTGCAA
AAAAGCATCTTGCTTTTGCAAGAAATAGTAACATCATTCAATATGCTTTCTTATTTACTAAAACCTT
GAAATAAAATTGTAAAACATCAAAAAAAAAAAAA
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FIG. 2. Nucleotide and deduced amino acid sequences of human IAP. Nucleotides are numbered on the right. Amino acids are numbered above the sequence, and the sequenced peptides are underlined with thin solid lines. The sites of potential asparagine-linked glycosylation are indicated by asterisks. Boxes indicate the putative signal sequence (nucleotides 9–65), the phosphate-binding serine active site (nucleotides 418–426), and a putative transmembrane region (nucleotides 1612–1656). The polyadenylylation consensus sequence AATAAA is underlined with a thick solid line. About 1.4 kb of the 3′ untranslated region are represented by parentheses and not shown.

Sequencing of IAP cDNA. On sequencing, clone λ8C proved to be a shortened version of λ13A at the 5′ end, starting at nucleotide 1026. It was not further characterized. The nucleotide sequence of clone λ13A is shown in Fig. 2 along with the deduced amino acid sequence of the protein. About 1.4 kb of the 3′ untranslated region that was sequenced is not included in the figure for editorial reasons. The 1.3-kb *Hin*dIII–*Hin*dIII fragment of clone λ13A was found to contain ≈300 base pairs of the λ DNA sequence fused to the poly(A) tail of the IAP cDNA insert, demonstrating that the *Eco*RI restriction site had been lost in the linker region at the 3′ end of the insert. Sequencing of the 2.1-kb *Eco*RI–*Hin*dIII fragment of clone λ13A showed that it probably contains the entire translated region.

The 5′ end of IAP cDNA shows a very short stretch of nucleotides (8 base pairs) followed by an ATG codon, most likely the translation initiation codon. The sequence representing the signal peptide apparently starts at nucleotide 9 and extends to nucleotide 65. If clone λ13A contains the entire translated region, then the codons representing the first two amino acids of mature IAP would correspond to nucleotides 66 to 71. Surprisingly, this short translated sequence is immediately followed by a long untranslated stretch (nucleotides 72–153) before the codon for the third amino acid of IAP (proline) appears. The rest of the translated region then follows in order. This insert, particularly because it came so early in the translated region, lent considerable confusion to interpretation of our data. Were it not for the availability of the peptide sequencing data, it would have been impossible to select the proper reading frame and identify the correct amino terminus of IAP. The peptide sequences shown in Fig. 2 agreed in all cases with the sequences deduced from cDNA sequencing. Though no splicing consensus sequence could be found, the insertion between the first two codons of IAP and the remainder of the coding region may represent an unspliced intron. This can be verified by cloning and sequencing the IAP gene. Alternatively, the insertion may be an artifact of our cloning procedure. However, computer sequence comparisons showed no homology with the DNA of λ phage, pBR322, or M13, or with any other region of IAP cDNA. It should be noted that cDNA clones of the same PLAP variant, cloned by Millan (5) and Henthorn *et al.* (6), differ in that the latter contains an unexpected repeating insert in the 3′ untranslated region, which was taken to be an artifact of cloning (6).

Comparison of IAP and PLAP. The 5′ end of IAP cDNA has a much shorter untranslated region than any reported for PLAP clones (4–6). It remains to be seen whether our IAP cDNA contains the complete 5′ region. Our IAP cDNA insert is larger (≈3.1 kb) than the largest PLAP cDNA that has been reported (4). The three nucleotides immediately following the termination codon are the same for IAP and PLAP cDNA (4–6). However, the remainder of the 3′ untranslated regions are quite different. Kam *et al.* (4) found several polyadenylylation consensus sequences in their PLAP clone; Millan (5) found at least two such sequences. Only one consensus sequence appears in the IAP cDNA in proximity to the polyadenylylation sequence. These differences in the 3′ untranslated regions should make it possible to prepare excellent probes to differentiate the transcripts of the two isozymes.

As can be seen in Fig. 3, the overall homology of the protein sequences of IAP and the PLAP variant reported by Kam *et al.* (4) is 86%. The N-linked asparagine glycosylation sites are conserved and there is one conservative change at the active (phosphate-binding) site. The carboxyl terminus of PLAP shown in Fig. 3 (residues 508–513) is entirely conserved in all the reported PLAP variants. However, only two of these six residues are conserved in IAP. The large hydrophobic region indicated by Kam *et al.* (4) (residues 491–507), which suggests a possible membrane-spanning region of PLAP, is almost entirely conserved in IAP. There is, however, a deletion of 12 nucleotides in the IAP cDNA, representing residues Gly-Arg-Ser-Val (residues 489–492) of PLAP. IAP also contains an additional cysteine in this region (residue 483). These differences at the carboxyl terminus may

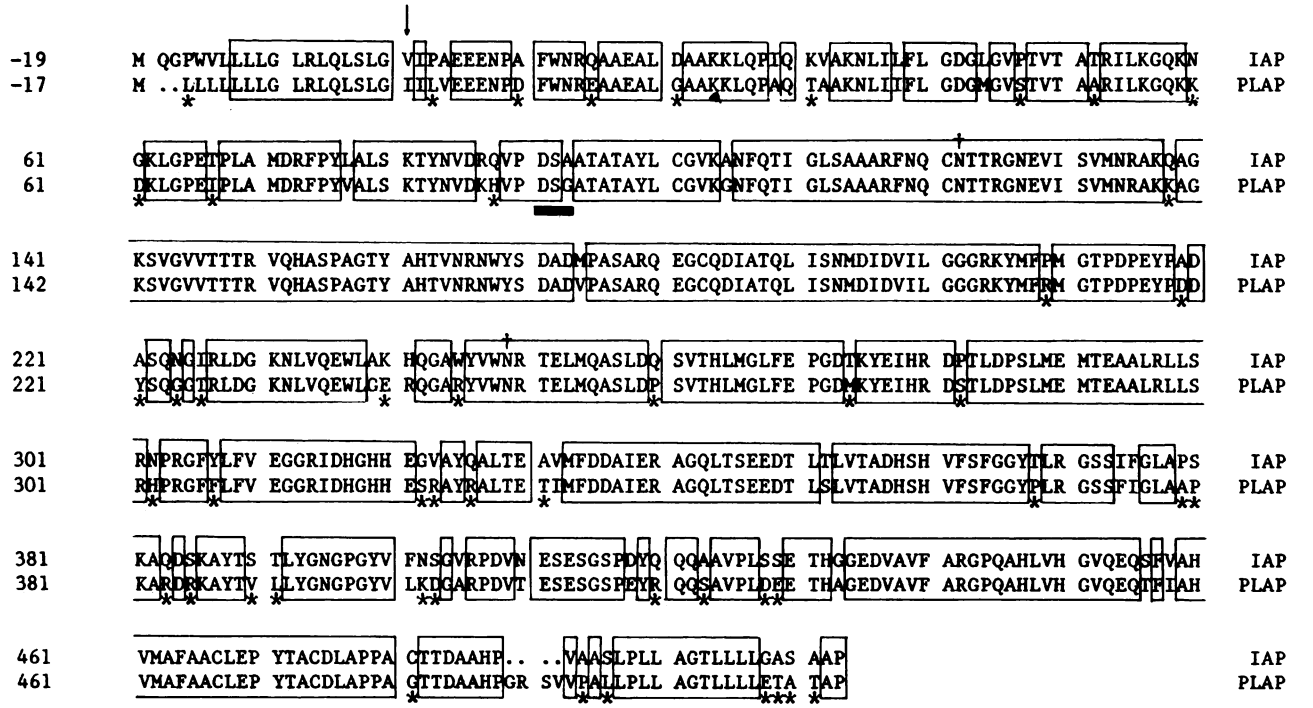

FIG. 3. Comparison of the deduced amino acid sequences of IAP and PLAP. Standard one-letter amino acid symbols are used. Boxed regions represent areas of homology between the two enzymes. Asterisks represent nonconservative changes. The arrow indicates residue 1 of the mature forms of these enzymes. Crosses represent putative sites of N-glycosylation (residues 122 and 249). The underlined residues, 91–93, are the putative active sites of the alkaline phosphatases.

be significant. In the proposed mechanism for anchoring alkaline phosphatase to the plasma membrane suggested by Low *et al.* (15), an aspartic acid or glutamic acid residue near the carboxyl terminus of the enzyme condenses with the ethanolamine end of a phosphatidylinositol molecule. In the process, a short carboxyl-terminal peptide sequence is released. If the putative condensing enzyme required for such a reaction is fairly specific, then IAP and PLAP may differ in their mode of attachment to the plasma membrane. In that regard, human PLAP is released from the plasma membrane by phosphatidylinositol-specific phospholipase C (16), whereas the rat intestinal enzyme is not released in this manner (17). The differences in the carboxyl termini of the two isozymes can be exploited to investigate the mechanism for anchoring alkaline phosphatase to the plasma membrane.

The comparison of IAP and PLAP shown in Fig. 3 is only with the variant of PLAP reported by Kam *et al.* (4), according to Henthorn *et al.* (6) an as yet uncharacterized variant. Table 1 summarizes the differences, at the protein level, among all the reported variants of PLAP and compares them to the corresponding residues in IAP. This comparison shows that IAP has greater homology with variant I (89%) than with any of the other variants. Based on immunological similarities, it has been suggested (18) that PLAP, which appears only in higher primates, evolved from IAP, which is apparently present in all mammals. The comparisons shown in Table 1 further support this relationship. Since PLAP variant I shows the greatest homology to IAP, it may be more closely related to the progenitor of PLAP than any of the other PLAP variants.

Due to the great homology between PLAP and IAP, polyclonal antisera to PLAP crossreact with IAP and vice versa (18). Harris (19) has generated monoclonal antibodies that distinguish between the two isozymes. Based on the homology of the two amino acid sequences, we estimate that many hybridoma clones had to be screened to find those that distinguished IAP from PLAP. It is interesting, therefore, that the synthetic peptide Ile-Ile-Pro-Val-Glu-Glu-Glu-Asn-Pro (representing the amino terminus of PLAP) plus the linker Phe-Gly-Cys, when coupled to keyhole limpet hemocyanin, yielded polyclonal antisera that are highly specific for PLAP and do not crossreact with IAP (unpublished observations). The corresponding sequence for IAP is Val-Ile-Pro-Ala-Glu-Glu-Glu-Asn-Pro. The specific antisera, together with the cDNA clones and the established primary amino acid sequences will be important and very useful in further studies of the alkaline phosphatases.

Table 1. Differences in amino acid residues of PLAP variants and comparison with IAP

Amino acid residue	PLAP			IAP
	Type I*	Type III*	Kam *et al.*†	
3	Pro	Leu	Leu	Pro
44	Met	Val	Met	Leu
209‡	Arg	Arg	Arg	Pro
239	Ala	Ala	Gly	Ala
240	Lys	Lys	Glu	Lys
241	Arg	His	Arg	His
255	Gln	Arg	Gln	Gln
263	Thr	Ala	Thr	Thr
302	Asn	Asn	His	Asn
362	Tyr	Cys	Tyr	Tyr
372	Ser	Gly	Ser	Ser
374	Ile	Ile	Phe	Ile
375	Phe	Phe	Ile	Phe
379	Pro	Pro	Ala	Pro

*From data of Henthorn *et al.* (6).
†From data of Kam *et al.* (4).
‡Protein sequence of variant of Millan (5) is identical to type I except for a proline at residue 209 instead of an arginine. The incomplete PLAP sequence report by Ovitt *et al.* (7) is identical to the corresponding portion of type I.

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