Vol. 156, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1988 Pages 563-569

EVIDENCE FOR THE EXPRESSION OF A PRIMITIVE INTESTINAL-LIKE ALKALINE PHOSPHATASE IN THE INTESTINAL 407 CELL LINE

Kazuyuki Hirano[1,*], Tomikazu Kusano[1], Kentaro Asai[1], Kyozo Hayashi[1], Torgny Stigbrand[2], Shiro Iino[3] and Jose L Millan[4]

[1]Gifu Pharmaceutical University, 6-1 Mitahorahigashi 5-chome, Gifu 502, Japan,

[2] Department of Physiological Chemistry, University of Umea, S-901 87 Umea, Sweden,

[3]Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Tokyo 113, Japan

[4]Cancer Research Center, La Jolla Cancer Research Foundation, La Jolla, California 92037

Received July 25, 1988

SUMMARY: Intestinal-like alkaline phosphatase was found to be expressed in the intestinal 407 cell line. This enzyme was identified by use of monoclonal antibodies specific for human placental (H_7 and HPMS-1) and intestinal alkaline phosphatase (2HIMS-1 and 2HIMS-3) separately. Purification of this isozyme by use of two different monoclonal antibody immunoaffinity chromatographies demonstrates a single protein band on SDS-polyacrylamide gel electrophoresis indicating that this enzyme is not formed as a heterodimer. The apparent monomer subunit molecular weight and the dimer molecular weight of this isozyme were determined to 70000 and 160000, respectively. The enzyme is a homodimer according to molecular weight determinations. Furthermore, this isozyme is neuraminidase sensitive and comparatively heat stable, properties also characteristic for the placental enzyme.

Our data suggest that the intestinal-like alkaline phosphatase in the intestinal 407 cell line displays properties intermediate of the intestinal and placental isozymes which may reflect the existence and reexpression of a new primitive isozyme.

INTRODUCTION: Human alkaline phosphatases (E.C.3.1.3.1) comprise a complex group of isozymes encoded for by at least four different genes (1). cDNA's encoding three isozymes have been cloned and sequenced (2-5) as well as the gene coding for germ cell alkaline phosphatase (6), revealing 52% homology between the

Abbreviation used: MICA, monoclonal immunocatalytical assay.

* To whom correspondence should be addressed.

0006-291X/88 $1.50

tissue-unspecific and placental alkaline phosphatase molecules, and 80% homology between the intestinal and the placental alkaline phosphatases at the amino acid level. The placental alkaline phosphatase shows a unique degree of allelic polymorphism and a number of alleles have been described at the placental gene locus (7). Random combinations of subunits result in more than 40 phenotypes (8). Placental alkaline phosphatase was one of the first proteins found to be ectopically expressed by cancer cells (9). Intestinal alkaline phosphatase also appears in at least two different forms: one is the adult intestinal alkaline phosphatase and the other is the fetal intestinal alkaline phosphatase (10).

In the past decade, two reports have been presented dealing with hybrid types of alkaline phosphatases as primitive isozymes (11, 12). It is however not yet confirmed that hybrid enzymes exist as long as polyclonal antibodies for placental or intestinal alkaline phosphatases are used to characterize the hybrid enzymes because these enzymes have been shown to share several antigenic determinants. In this paper, we present evidence for existence of a primitive intestinal-like alkaline phosphatase expressed in a intestinal 407 cell line.

MATERIALS AND METHODS

Monoclonal antibody : Monoclonal antibodies were prepared according to the method of Köhler and Milstein (13). H_7 and HPMS-1 monoclonal antibodies are specific for the placental alkaline phosphatase, 2HIMS-1 for the adult and fetal intestinal alkaline phosphatases, 2HIMS-3 for the adult intestinal alkaline phosphatase and HLMS-1 for tissue-unspecific alkaline phosphatase (14-16). The monoclonal antibodies were used after Protein A-Sepharose CL-4B purification, as earlier described (14).

Purification of the intestinal-like alkaline phosphatase : Cultured intestinal 407 cells were homogenized in 1 volume of butanol and 3 volumes of Tris-HCl buffer, pH 7.5, containing 10 µM $MgCl_2$ and $ZnCl_2$ and 0.02% NaN_3. The water-phase was dialyzed against the above buffer. After centrifugation, the dialyzed sample was applied on a HPMS-1 column and washed with 50 mM of Tris-HCl, pH 8.0, containing 0.9% NaCl. The enzyme was eluted with 0.2 M Na_2CO_3 containing 0.5 M NaCl. The active fractions were neutralized by addition of 0.1 M acetic acid and dialyzed against 50 mM Tris-HCl buffer, pH 8.0, containing 0.5 M NaCl. The enzyme was then, in a second cycle, applied on a 2HIMS-1 column and the intestinal-like alkaline phosphatase was eluted by use of the same procedure.

Monoclonal immunocatalytical assays (MICAs) : MICAs were performed as described previously (15, 16). Briefly, each monoclonal antibody bound to paper discs was used to trap the isozyme and the activity on the paper disc was determined using phenylphosphate or 4-methylumbelliferyl phosphate as substrate.
SDS-polyacrylamide gel electrophoresis : 9% polyacrylamide gel was used according to Laemmli (17).

RESULTS AND DISCUSSION

The intestinal 407 cells were expected to express the fetal intestinal alkaline phosphatase. However this cell line expresses several phosphatase isozymes including the intestinal-like alkaline phosphatase as well as the placental and tissue-unspecific alkaline phosphatase as judged from the electophoretic mobility and the reactivity with the monoclonal antibodies. The levels of expressed alkaline phosphatase isozymes were determined by the MICAs as shown in Table I. The intestinal-like alkaline phosphatase is the main enzyme in terms of activity and this enzyme reacts with the monoclonal antibody 2HIMS-1 but not with 2HIMS-3. Furthermore, the electrophoretic mobility of the intestinal-like enzyme is clearly different from that of the adult and fetal intestinal alkaline phosphatases, suggesting the existence of a new type of intestinal-like alkaline phosphatase in this cell line. Existence of hybrid-type alkaline phosphatases (heterodimers) has been reported in KB-cells, FL-cells and the fetal intestine, respectively (11, 12). However, the mixture of placental and intestinal-like alkaline phosphatases presents the same immunochemical reactivity with polyclonal antibodies as those of the hybrid enzymes if not monoclonal antibodies specific for each isozyme are used as discriminatory tools. Purification of this new type of

Table I. Alkaline phosphatase isozymes in Intestine 407 cells

Isozyme	Antibody	Activity (IU/mg protein)
Placental alkaline phosphatase	HPMS-1	0.449
Liver alkaline phosphatase	HLMS-1	0.165
Intestinal alkaline phosphatase	2HIMS-1	6.830
Intestinal alkaline phosphatase	2HIMS-3	0.600

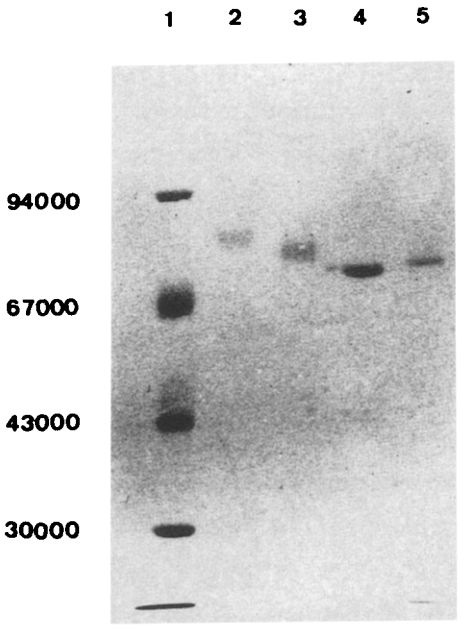

Figure 1. SDS-polyacrylamide gel electrophoresis of different purified alkaline phosphatases.
9% polyacrylamide gel was used and the proteins were stained by Coomassie Brilliant Blue R-250. Lane 1; marker proteins, lane 2; intestinal alkaline phosphatase, lane 3; meconial alkaline phosphatase, lane 4; placental alkaline phosphatase, lane 5; intestinal-like alkaline phosphatase in the intestinal 407 cells.

intestinal-like alkaline phosphatase is necessary to further elucidate the structure of this enzyme. The new enzyme was purified by use of two different monoclonal antibodies coupled to CH-Sepharose 4B columns. Fig. 1 shows the SDS-polyacrylamide gel electrophoretic pattern of the purified intestinal-like alkaline phosphatase from the intestinal 407 cells. The fractions which bind to the two monoclonal antibody columns demonstrate a single protein band (Mr 70000), clearly different from the placental and intestinal enzyme and the molecular weight of this intestinal-like alkaline phosphatase was calculated to 160000 from Sephadex G-200 gel filtration data. These results strongly suggest that the new type of intestinal-like alkaline phosphatase in the 407 cells may be a primitive enzyme and not a hybrid-type alkaline phosphatase. This intestinal-like alkaline phosphatase was furthermore confirmed to be neuraminidase sensitive (data not shown). The immunoreactivity data of the new intestinal-like

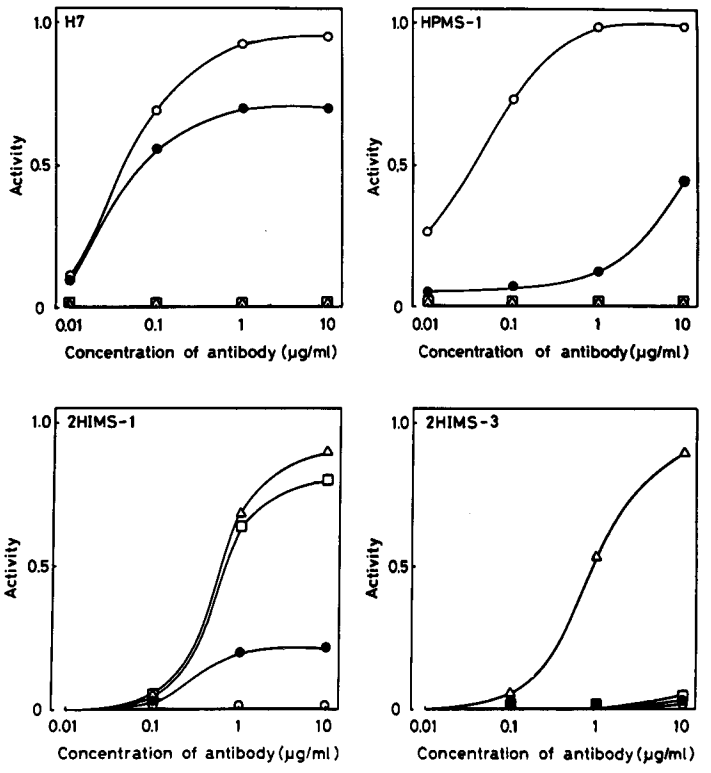

Figure 2. Reactivities with different monoclonal antibodies with the intestinal-like alkaline phosphatase from intestinal 407 cells.
100 microliter of an anti-mouse IgG antibody was coated in the 96 wells immunoplate at 37°C for 3 h followed by washing three times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 and 0.05% sodium merthiolate (PBS-Tween). The wells were blocked with 300 μl of 1% bovine serum albumin dissolved in PBS containing 0.05% sodium merthiolate at 4°C overnight and then the wells were washed with 400 μl of PBS-Tween 20. 100 μl of the indicated monoclonal antibody, dissolved in 0.1%-PBS, was added to each well and allowed to stand for 3 h at 37°C. The wells were washed three times with 10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl 0.05% Tween 20 and 0.05% sodium merthiolate. 100 μl of alkaline phosphatase (70 IU/L) dissolved in 0.1% BSA-10 mM Tris-HCl buffer, pH 7.4, containing 0.02% sodium azide was added to each well and allowed to stand for 3 h at 37°C. The activity was determined in 200 μl of 2.7 mM p-nitrophenylphosphate dissolved in 0.1 M ethanolamine-HCl buffer, pH 9.8, containing 0.1 mM $MgCl_2$.
o: placental alkaline phosphatase, △: intestinal alkaline phosphatase, □: meconial alkaline phosphatase, ●: intestinal-like alkaline phosphatase.

alkaline phosphatase with the monoclonal antibodies indicated that this enzyme reacts with both the placental and intestinal specific monoclonal antibodies (H_7, HPMS-1 and 2HIMS-1), but not with the adult intestinal specific monoclonal antibody (2HIMS-3) as shown in Fig. 2. Therefore the new enzyme probably contains structures reminding of human placental and fetal intestinal

alkaline phosphatases but not of adult intestinal alkaline phosphatase. The heat stability of the new enzyme is also intermediate to the placental and fetal lintestinal alkaline phosphatases.

The above results may give rise to speculations that the intestinal-like enzyme in intestinal 407 cells is a primitive alkaline phosphatase with common determinants of both the human placental and fetal intestinal alkaline phosphatases and the translational product of a gene intermediate in the evolution of the placental and fetal intestinal alkaline phosphatase genes. It is still unknown which regulatory mechanisms are involved in the expression of this enzyme. The identification of potential remnants of evolutionary products is of significant importance for the elucidation of the development of the complex phosphatase isozyme group in man.

ACKNOWLEDGMENT

This work was supported in part by a Grant-in-Aids for General Scientific Research from the Ministry of Education, Science and Culture, Japan.

REFERENCES

1. Fishman, W.H. (1974) Am. J. Med. **56**, 617-650.
2. Kam, W., Clauser, E., Kim, Y.S., Kan, Y.W. and Rutter, W.J. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 8715-8719.
3. Millan, J.L. (1986) J. Biol. Chem. **261**, 3112-3115.
4. Berger, J., Garattini, E., Hua, J.-C. and Udenfriend, S. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 695-698.
5. Weiss, M.J., Henthorn, P.S., Lafferty, M.A., Slaughter, C., Raducha, M. and Harris, H. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 7182-7186.
6. Millan, J.L. and Manes, T. (1988) Proc. Natl. Acad. Sci. U.S.A. in press.
7. Stigbrand, T., Millan, J.L. and Fishman, W.H. (1982) The geneticbasis of alkaline phosphatase isozyme expression. In Isozymes: Current Topics in Biological and Medical Reserch. Vol. 6, A.R. Liss, pp 93-117.
8. Beckman, L., Bjorling, G. and Christodoulou, C. (1966) Acta Genet. Basel **16**, 59-73.
9. Fishman, W.H., Inglis, N.R., Green, S., Anstiss, C.L., Gosh, N.K., Reif, A.E., Rustigian, R., Krant, M.J. and Stolbach, L.L. (1968) Nature **219**, 697-699.
10. Sugiura, M., Hirano, K., Iiizumi, Y., Miyazaki, J., Miki, K., Iino, S., Suzuki, H. and Oda, T. (1981) Chem. Pharm. Bull. **29**, 3660-3666.

11. Singer, R.M. and Herz, F. (1978) J. Natl. Cancer **60**, 573-577.
12. Behrens, C.M., Enns, C.A. and Sussman, H.H. (1983) Bichem. J. **211**, 553-558.
13. Köhler, G. and Milstein, C. (1975) Nature **256**, 495-497.
14. Millan, J.L. and Stigbrand, T. (1983) Eur. J. Biochem. **136**, 1-7.
15. Hirano, K., Iiizumi, Y., Hayashi, Y., Tanaka, T., Sugiura, M., Hayashi, K., Lu, Z-D. and Iino, S. (1986) Anal. Biochem. **154**, 624-631.
16. Hirano, K., Matsumoto, H., Tanaka, T., Hayashi, Y., Iino, S., Domar, U. and Stigbrand, T. (1987) Clin. Chim. Acta **166**, 265-273.
17. Laemmli, U.K. (1970) Nature **222**, 680-681.