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Expression of alkaline phosphatase in murine lymphoma cells

Jean Harb, Fabienne Vavasseur, Corinne Chadéneau,
Marc Denis and Khaled Meflah

U.F.R. de Médecine, Laboratoire de Biochimie Médicale (CJF INSERM 90-11)
1, rue Gaston Veil - 44035 Nantes Cedex, France

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SUMMARY : Alkaline phosphatase (ALP) was secreted and expressed at the cell surface of the lymphoma A/63-2 cell line but not on another clone A/63-1 deriving from a single thymoma (A/63) induced by a wild-type Abelson-Moloney viral complex. The enzyme was heat-sensitive and strongly inhibited by L-p-bromotetramisole and L-homoarginine but not by L-phenylalanine. All these data indicated that this enzyme was most likely identical to the L/B/K ALP isoenzyme. Southern blot analysis showed that neither amplification nor polymorphism were responsible for the high expression of the ALP gene observed in A/63-2 cells. On the opposite, the mRNA transcripts of ALP were only detected in A/63-2 cells indicating that a modulation of the ALP gene transcription occured which could be due to the insertion of the v-abl gene within or near the 5'-flanking region of the ALP promotor in A/63-2 cells. Butyrate strongly increased both the secretion and the expression of the enzyme on A/63-2 cell surface. This induction was strongly inhibited by cordycepin, an RNA biosynthesis inhibitor, and at a lesser degree by cycloheximide, a translation inhibitor suggesting that butyrate induction occurs both at the transcriptional and the translational level.

Alkaline phosphatases (ALPs) (orthophosphoric monoesters phosphohydrolases, alkaline optimum), EC 3.1.3.1., are a group of glycophosphatidylinositol anchored (1) membrane metalloenzymes. They hydrolyze a broad range of monophosphate esters at alkaline pH. In human, four distinct ALP genes encode the intestinal, placental, placental-like, and Liver/ Bone/Kidney (L/B/K) forms of the enzyme (2-6). In mouse, three genetic loci, Akp -1, -2, and -3 have been identified and proposed to encode different tissue isoforms of ALPs. The L/B/K and the placental isoforms are encoded by the Akp-2 locus located on chromosome 4 (7-9), while the intestinal isoform of murine ALPs is encoded by the Akp-3 gene which maps to the proximal portion of chromosome 1 (10, 11). The three isoenzymes of ALPs differ by their molecular weight, thermal stability, immunological properties and sensitivity to inhibitors (12-14).

ALP enzymatic activity is found in various tumors and tumor cell lines such as teratocarcinomas (15-16), choriocarcinomas (17-18) and osteosarcomas (19).

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Expression of the ALP is considered as one of the many identified markers of malignancy and cell surface phenotypic changes (20). It has been reported that following in vitro malignant transformation of a variety of cells including those of human placental origin, the activity of ALP was modified. A reduced ALP activity has been observed in cells transformed by Rous sarcoma virus (21) or Simian virus 40 (22). On the opposite, tumorigenicity correlated with a high level of ALP expression in a fibroblastic cell line (23). In addition, the enzyme activity was differently expressed in several cancer cell lines differing by their origin such as in osteosarcomas cell lines (24).

In our laboratory, we have produced a syngeneic model to study metastasic processes. This model consists of 2 murine thymic lymphoma (A/63-1 and-2) induced by intraperitoneal injection of a newborn mouse with an Abelson-Moloney virus complex (25). The two lymphoma cell clones, derived at an early stage of T-cell differentiation, were selected for their different colonizing capacities. Only the A/63-1 clone colonizes liver.

Here, we observed that only the A/63-2 clone exhibits ALP activity at the cell surface as well as in soluble form in the culture medium. The characterization and effect of butyrate on this enzyme as well as the origin of its expression were investigated.

MATERIALS and METHODS

Reagents : RPMI 1640 was purchased from Gibco (Grand Island, NY). Fetal calf serum (FCS), phosphate-buffered saline (PBS), L-glutamine and antibiotics were from Seromed (Noisy-le-Grand). n-Butyric acid (sodium salt), cycloheximide, cordycepin, thymidine, L-phenylalanine, L-hormoarginine, phospholipase A2 (bee venom) and phospholipase D (Cabbage) were from Sigma (St Louis, MO). PI-PLC (Bacillus cereus) was from Boehringer (Mannheim, FRG). L-p-bromotetramisole was from Aldrich Chemical (Millawkee, WI).

Cell lines and culture : Tumor induction and clones establishment were realized as previously desribed (25). Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 50 mM 2-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. The cells were routinely tested for Mycoplasma (26) and found to be free of contamination.

Enzyme assays : ALP activity was determined by the optimized standard method using a Boehringer kit. The hydrolysis of p-nitrophenylphosphate (27) at pH 9.5 and 37°C was monitored at 405 nm. Specific activity was expressed in nmol of substrate hydrolyzed in 1 min. per mg of protein. ALP thermostability was determined by preincubating duplicate aliquots with 0.1 ml of triethanolamine buffer pH 9.5 at 56°C during 25 min. The percentage of activity remaining was computed from controls kept with buffer at 4°C.

Inhibition studies were done on both membrane bound and secreted ALP. The specific inhibitors tested were (range of final concentrations in parenthesis): L-phenylalanine (1-40mM), L-Homoarginine (1-100mM), L-Bromotetramisole (0.005-0.2mM). The results were expressed according to Goldstein and Harris (12) as the concentration of inhibitor required to produce 50% inhibition ([I] 50).

To study the response of both cell lines to butyrate, 10^6 cells of each line were allowed to grow in 25 cm^2 flask during 24 hours before adding the inducer. Butyrate

was used at final concentration of 2 mM. Every 24 hours, a sample of the cell suspension was removed to measure the ALP activity.

The effect of inhibitors of macromolecular synthesis (thymidine, cordycepin, cycloheximide) on ALP induction by butyrate was studied by adding the agents together with the butyrate. The ratio of the specific activity of the experimental cultures to the controls was referred to as the "induction ratio".

PI-PLC treatment : Samples of 10^7 A/63-2 cells were incubated in 200 µl of 70 mM triethanolamine buffer pH 7.4, 0.25 M sucrose at 37°C in the presence or absence of 25 mU PI-PLC (28). At different times, the suspension was centrifuged at 100,000g, and ALP activity was measured in both the supernatant and the pellet. Similarly, the effect of phospholipase A2 (6 U) and phospholipase D (1 U) was performed as controls.

Protein assay : Protein concentrations were determined by the BCA standard method using the Pierce kit (Rockford, ILL) according to the manufacturer's recommandations.

RNA preparation and detection of ALP RNA transcripts : Total cellular RNA was extracted from A/63-1 and A/63-2 cells using the guanidine thiocyanate method (29). 20 µg of total RNA were combined with 5 µl deionized formamide, 1.5 µl formaldehyde, and 1 µl of 10X MOPS buffer (30) heated at 68°C for 5 min, and then cooled on ice. The samples were loaded onto 1% agarose minigel containing 2.2 M formaldehyde and electrophoresed for 3h at 50 V. The RNA was transferred to a Photogene™ nylon membrane (BRL, Gaithersburg, MD). Following the transfer, RNA was crosslinked to the membrane by U.V. irradiation for 5 min (31). The amount of RNA per lane on the filters was judged to be equal by ethidium bromide staining of the ribosomal bands (not shown). The nylon filter was hybridized to a 1 kb fragment of the *mouse placental alkaline phosphatase cDNA plasmid* pSPmp1 (8) *kindly provided by* Dr Mintz (Philadelphia, U.S.A) labelled with biotin-14-dATP by random priming. Following hybridization, the membrane was washed with 1X SSC/1% SDS (1x SSC=0.15 M NaCl/0.015 M sodium citrate) at 65°C, 0.5X SSC/0.1% SDS at 50°C, 2X SSC at room temperature, and then incubated with streptavidin-alkaline phosphatase conjugate (BRL) according to the Manufacturer's recommandations. The membrane was exposed to an Amersham Hyperfilm-MP for 20 min at room temperature.

Southern blot analysis of A/63-1 and A/63-2 DNA : High molecular weight DNA prepared from A/63-1 and A/63-2 cells using a standard method (30) was digested with *Eco*RI, *Bam*HI, or *Hind*III, electrophoresed on a 0.8% agarose gel (10 µg/lane) and transferred to an Hybond-N nylon membrane (Amersham, Littlte Chalfont, UK). The membrane was hybridized with ^{32}P-labelled mouse placental ALP probe (10^9 cpm/µg) in 10% dextran sulfate, 2X SSC and 5% SDS for 18 hours at 65°C. After hybridization, the membrane was washed sequentially with 1X SSC/0.1% SDS at 65°C, 0.5X SSC/0.1% SDS at 65°C and 0.1X SSC/0.1% SDS at 65°C and autoradiographed for 2 days at -80°C.

RESULTS

ALP activity in thymocytes, A/63-1 and A/63-2 cells. The ALP activity was undetectable in mouse thymocytes, as well as in A/63-1 cell line. However, the activity was 4 nmol/min/mg protein in A/63-2 cell line. This activity was 25 nmol/min/mg in purified plasma membranes. In addition, A/63-2 cells secreted ALP in culture medium which was 0.05 nmol/min/ml when cells were at 2 x 10^6 cells /ml. A/63-1 cells did not secrete any ALP activity.

Characterization of the A/63-2 cells ALP. Table 1 summarizes the effect of several inhibitors on ALP activity. The enzyme was completely heat -sensitive

Table 1 . Concentrations of inhibitors producing 50% inhbition of A/63-2 cells ALP

Inhibitor	A/63-2 cells enzyme	Term placental enzyme [a]	Intestinal enzyme [a]	L/K/B enzyme [a]
L-phenylalanine	> 50	4.5	3	> 50
L-homoarginine	3	> 50	> 50	3.5
L-bromotetramisole	0.01	0.6	3	0.01
Température (56°C)	sensitive	resistant	sensitive	sensitive

Assays were performed in duplicate as described under "Materials and Methods". At least five concentrations of each inhibitor were used. Estimates of [I] 50 were obtained from graphs depicting 100/percentage of activity vs concentration of inhibitor. The heat treated enzyme was realized at 56°C during 20 min. All values are [I] 50 expressed in mM.

[a] Herz, F. (13)

indicating that it does not correspond to the placental isoenzyme. The inhibition profile showed that the enzyme was strongly sensitive to L-p-bromotetramisole and L-homoarginine but resistant to L-phenylalanine. In addition, the enzyme was not recognized by a monoclonal antibody against the intestinal isoenzyme (not shown). All these observations indicate that the A/63-2 ALP is the L/B/K isoenzyme. The secreted isoenzyme exhibited similar properties (not shown). Finally, as most of ALP enzymes, the A/63-2 was released from the plasma membrane by PI-PLC treatment (Fig. 1). One should note that a portion of this enzyme was released in the absence of PI-PLC; however, the percentage of the released enzyme did not increase with incubation time. When phospholipase A2 or phospholipase D were used, a percentage of the enzyme equivalent to the control was released (results not shown).

Expression levels of alkaline phosphatase mRNA in both clones. The levels of ALP steady-state mRNA were examined to determine the basis of the observed differential expression of ALP activity in both cell lines. We performed a Northern blot using, as a probe, a mouse placental ALP cDNA which presents high homology with the L/B/K isoenzyme (8, 9). Figure 2 shows that no detectable ALP mRNA was observed in A/63-1 cell line, whereas ALP transcripts (2.5-kb) were abundantly expressed in the A/63-2 cell line.

Southern blot analysis of A/63-1 and A/63-2 genomic DNA. DNA blotting analysis was performed using a 1-kb fragment of the mouse placental ALP cDNA and is shown in figure 3. After digestion with *Eco*RI, *Bam*HI or *Hind*III, bands of the same size and intensity for both clones appeared on the autoradiogram. Thus, neither amplification nor polymorphism of the ALP gene were observed in the A/63-2 cell line. The bands obtained after each kind of enzyme digestion corresponded to those observed in mouse thymus DNA (9).

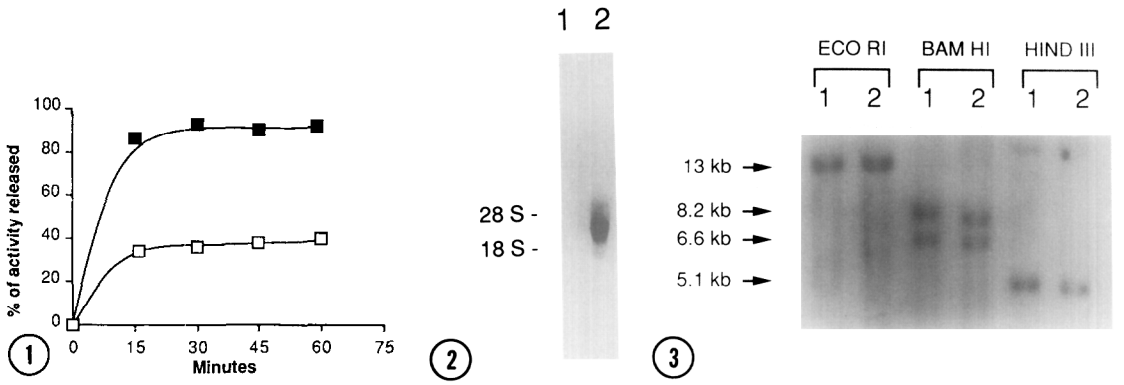

Figure 1 . Effect of PI-PLC on the ALP releasing from A/63-2 cells.

10^7 A/63-2 cells were incubated in the absence (□) or the presence (■) of 25 mU of PI-PLC as described under "Materials and Methods". Results are expressed as percentage of ALP recovered in the supernatants and are means of two experiments.

Figure 2. Northern blot analysis of A/63-1 and A/63-2 RNA.

Total RNA prepared from A/63-1 cells (**1**) and A/63-2 cells (**2**) was analyzed using a portion of the mouse placental alkaline phosphatase cDNA as a probe, as detailed under "Materials and Methods". On the left side are indicated the positions of the ribosomal 18S and 28S RNA.

Figure 3 . Southern blot analysis of A/63-1 and A/63-2 DNA.

DNA prepared from A/63-1 cells (**1**) or A/63-2 cells (**2**) was digested with *Eco*RI, *Bam*HI, or *Hind*III, size fractionated on a 0.8% agarose gel, transferred to a nylon membrane and hybridized with ^{32}P-labelled mouse placental alkaline phosphatase probe. The size of the different fragments are shown on the left hand side.

Effect of butyrate on ALP biosynthesis in both clones. To determine whether the A/63-1 ALP biosynthesis could be stimulated or not, we tested the effect of butyrate on both cell clones. As shown on figure 4A, the ALP specific activity of A/63-2 control

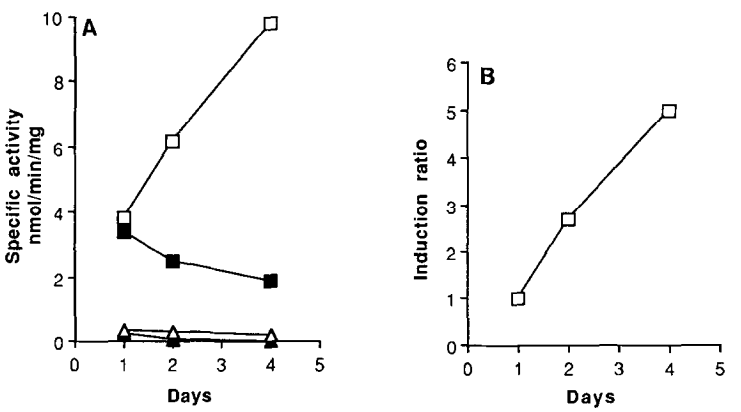

Figure 4 . Effect of butyrate on the ALP biosynthesis of A/63-1 and A/63-2 cells.

(A) Cells were grown 24 hours before adding 2 mM butyrate in the culture medium. After the indicated time, samples of the cell suspensions were removed to determine ALP activity. (▲) A/63-1 cells control; (△) A/63-1 cells treated with butyrate ; (■) A/63-2 cells control ; (□) A/63-2 cells treated with butyrate. (B) Data were plotted to determine the induction ratio of A/63-2 cells. Results are means of two experiments.

Table 2 . Effect of thymidine, cordycepin and cycloheximide on induction of ALP by butyrate in A/63-2 cells

Additions to culture medium	Specific activity	Activation ratio
None	2.9	1
Butyrate (2 mM)	12.2	4.20
Butyrate + Cordycepin (0.2 mM)	5.27	1.80
Butyrate + Cycloheximide (2 μM)	7.13	2.45
Butyrate + Thymidine (2 mM)	15.2 [a]	5.22

10^6 cells were allowed to grow in 25-cm^2 flask during 24 hours before adding butyrate or butyrate + different inhbitors. After 72 hours, cells were harvested and ALP activity measured. The specific activities are expressed as nmol of p.nitrophenol liberated / min / mg protein.

[a] Thymidine alone induced activation of ALP biosynthesis.

cells slightly decreased with time probably because some cells died after 4 days of culture in the same medium. However, in the presence of butyrate, which decreases cell growth, the ALP biosynthesis strongly increased after a lag-time of 48 hours, reaching 10 nmol/min/mg protein after 4 days of culture. In the case of A/63-1, a slight increase of the activity was induced by butyrate within 24 hours, but disappeared during the last days. Four days after addition of butyrate, the specific activity of ALP in A/63-2 cells was 50 fold higher than in A/63-1 cells. The "induction ratio" of ALP in A/63-2 increased from 1 at day 1 to 5 at day 4 (Fig.4B).

We tested the effect of several inhibitors of macromolecules biosynthesis on the induction of ALP by butyrate. The "induction ratio" of ALP biosynthesis was reduced from 4.2 to 1.8 in the presence of a RNA synthesis inhibitor, cordycepin (0.2mM) and to 2.45 in the presence of 2.5 μM cycloheximide, a translation inhibitor. However, the DNA synthesis inhibitor, thymidine, had no effect (Table 2).

DISCUSSION

The two lymphoma cell clones used in the present study were derived from a single thymic tumor, induced by a wilde-type Abelson-Moloney viral complex. Hybridization study with probe K2 derived from v-abl, provided evidence for the clonality of both cell lines (25). The analysis of the expression of T-cell markers and of the pre-B-cell marker B220 as well as the study of the rearrangement of γT cell receptor genes showed that both clones were probably derived from the transformation of early T-cell precursors before or after the turning-on of the Thy-1.2 antigen by the thymic microenvironment (25).

In this study, we present evidence that only the A/63-2 clone expresses ALP on its surface and secretes it. Moreover, immunological and biochemical studies showed that this enzyme is most likely identical to the L/B/K ALP isoenzyme. The expression of such ALP isoenzyme has been described in SAOS-2 but not in TE-85 human osteosarcoma cell lines (24). As most of ALPs (1), the A/63-2 isoenzyme was released from plasma membranes by PI-PLC treatment, suggesting a phosphatidylinositol-glycan anchoring. Our results showed that the bound enzyme was totally released from the membrane by PI-PLC treatment suggesting the absence of other anchor types, as it has been previously depicted in the case of the human choriocarcinomas (18) or adenocarcinomas (32) enzymes. The fraction of enzyme released in the absence of PI-PLC could represent the pool of enzyme in the route to secretion in the culture medium.

Our results concerning the ALP gene in both clones showed that neither amplification nor polymorphism were responsible for the high expression of this gene in A/63-2 cell clone. The mRNA transcripts appearing only in A/63-2 cells were about 2.5-kb long and were identical in size to mouse placental ALP (8, 9). Recently, Terao et al. (9) showed differential expression of mRNA transcripts of ALP in F9 and 3T3 fibroblast cells, the 5'-flanking region containing the promotor being active in F9 but not in 3T3 cells. The 5'-flanking region contains general elements essential for transcription as well as determinants for cell specificity of transcription. Similarly, Latham et al. (23) observed enhanced expression of ALP mRNA in tumorigenic CGL4 but not in non tumorigenic CGL1 human fibroblastic cell lines. This could be due to a negative transcriptional regulator. Alternatively, regulation could occur indirectly by modulation of a positive transcriptional activator. We have shown that a v-abl probe hybridized with two bands in A/63-2 but not in A/63-1 or A/63 cells at 14 and 8-kb when DNA was digested by *Eco*RI (25). One can speculate that v-abl gene is inserted within or near the 5'-flanking region of the ALP promotor. This insertion could influence the transcription of the ALP gene.

Butyrate strongly increased the secretion and the expression of the enzyme on A/63-2 cell surface. Both the normally expressed and the induced isoenzyme had similar biochemical properties (sensitivity to inhibitors). The induction showed a lag-time of 48 hours. Brown et al. (33) showed that butyrate induction of L/B/K ALP was due to a c-AMP dependent protein kinase. The relatively slow rate of induction of ALP transcription by butyrate suggests that ongoing protein synthesis is required for the effect of the c-AMP dependent butyrate (34). This is confirmed by the inhibition of butyrate induction observed with cycloheximide, a protein biosynthesis inhibitor. However, since cordycepin, used as a tanscription inhibitor, also prevents ALP induction by sodium butyrate, the stimulation of ALP biosynthesis in A/63-2 cells also implies a modulation of transcription. The 1.8 induction fold observed in the absence of transcription most likely involves the modification of pre-existing protein (s) to a more active form (34). Clearly, the mechanisms of induction of ALP in A/63-2 cells by sodium butyrate occurs both at the transcriptional and the translational level.

Since the physiological functions of ALPs are not known, one can only speculate as to their potential role in carcinogenesis. The A/63-2 cells represented a link between L/B/K ALP and invasive properties. However, experiences are undertaken to explore the ALP expression in other clones differing by their metastatic capacities. Thus, we should then be able to provide experimental systems to directly ascertain its significance in the tumorigenic phenotype.

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