Journal of Cellular Biochemistry 76:420–436 (2000)

# Ecto-Alkaline Phosphatase Activity Identified at Physiological pH Range on Intact P19 and HL-60 Cells Is Induced by Retinoic Acid

**Renate J. Scheibe,**[1,2]* **Henning Kuehl,**[1] **Stefan Krautwald,**[1] **Joachim D. Meissner,**[2] **and Walter H. Mueller**[1]

[1]Zentrum Biochemie OE 4310, Medizinische Hochschule, 30623 Hannover, Germany
[2]Zentrum Physiologie OE 4220, Medizinische Hochschule, 30623 Hannover, Germany

**Abstract** The activity of membrane-bound alkaline phosphatase (ALP) expressed on the external surface of cultured murine P19 teratocarcinoma and human HL-60 myeloblastic leukemia cells was studied at physiological pH using *p*-nitrophenylphosphate (*p*NPP) as substrate. The rate of substrate hydrolysis catalyzed by intact viable cells remained constant for eight successive incubations of 30 min and was optimal at micromolar substrate concentrations over the pH range 7.4–8.5. The value of apparent $K_m$ for *p*NPP in P19 and HL-60 cells was 120 μM. Hydrolytic activity of the ecto-enzyme at physiological pH decreased by the addition of levamisole, a specific and noncompetitive inhibitor of ALP ($K_i$ P19 = 57 μM; $K_i$ HL-60 = 50 μM). Inhibition of hydrolysis was reversed by removal of levamisole within 30 min. Retinoic acid (RA), which promotes the differentiation of P19 and HL-60 cells, induced levamisole-sensitive ecto-phosphohydrolase activity at pH 7.4. After its autophosphorylation by ecto-kinase activity, a 98-kDa membrane protein in P19 cells was found to be sensitive to ecto-ALP, and protein dephosphorylation increased after incubation of cells with RA for 24 h and 48 h. Orthovanadate, an inhibitor of all phosphatase activities, blocked the levamisole-sensitive dephosphorylation of the membrane phosphoproteins, while (R)-(−)-epinephrine reversed the effect by complexation of the inhibitor. The results demonstrate that the levamisole-sensitive phosphohydrolase activity on the cell surface is consistent with ecto-ALP activity degrading both physiological concentrations of exogenously added substrate and endogenous surface phosphoproteins under physiological pH conditions. The dephosphorylating properties of ecto-ALP are induced by RA, suggesting a specific function in differentiating P19 teratocarcinoma and HL-60 myeloblastic leukemia cells. J. Cell. Biochem. 76:420–436, 2000. 

**Key words:** ecto-alkaline phosphatase; differentiation; HL-60 myeloid leukemia cells; P19 teratocarcinoma cells; retinoic acid

Alkaline phosphatases (ALPs; orthophosphoric-monoester phosphohydrolase; EC 3.1.3.1) are a group of membrane-bound phosphomonoesterases [Seargeant and Stinson, 1979b]. Mammalian ALPs are expressed as placental, placental-like, intestinal, and tissue nonspecific or liver/bone/kidney (L/B/K) isozymes exhibiting a characteristic distribution pattern in tissues [for a review, see Millán and Fishman, 1995]. ALP is a member of the class of proteins that are linked to the exterior of the cytoplasmic membrane via phosphatidylinositol-glycan anchor [Low and Saltiel, 1988; Micanovic et al., 1988]. The enzyme can be released by membrane-impermeable glycosylphosphatidylinositol-specific phospholipase C or D, which provided evidence for its ecto-orientation [Low and Finean, 1977; Nakamura et al., 1988]. Enzymes that can act on extracellular substrate are referred to as ecto-enzymes [DePierre and Karnovsky, 1974]. The physiological function of ALPs in normal tissues and in malignant processes is under continuous investigation. The L/B/K ALP isozyme has been associated with bone calcification [Harris, 1990; Morris et al., 1992; Whyte, 1989] and a lack of enzyme caused

Abbreviations used: ALP, alkaline phosphatase; L/B/K isozyme, liver/bone/kidney isozyme; PI-PLC, phosphatidylinositol-specific phospholipase C; *p*NPP, *p*-nitrophenylphosphate; PBS, phosphate-buffered saline; PVA, polyvinyl alcohol; RA, all-*trans* retinoic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

Stefan Krautwald is currently at Fraunhofer Institut, Hannover, Germany.

*Correspondence to: Renate J. Scheibe, Zentrum Physiologie OE 4220, Medizinische Hochschule, 30623 Hannover, Germany. E-mail: Scheibe.Renate@MH-Hannover.de

Received 20 May 1999; Accepted 27 July 1999

defective mineralization in hypophosphatasia [for a review, see Caswell et al., 1991]. Despite the generally admitted importance of ALP for mineralization, the wide distribution of the enzyme in mammalian tissues makes a restriction of its physiological function solely to the mineralization process unlikely.

Increased expression of ALP has been observed in various cancer cells; determination of ALP levels is frequently used in clinical medicine [for a review, see Millán and Fishman, 1995]. An understanding of the reasons underlying increased ALP activity in cancers may provide insight into the process of carcinogenesis and normal functions of the enzyme. ALP activities in several cancer cells can be induced by retinoic acid (RA) [Cope and Wille, 1989; Ng et al., 1988, 1989; Reese et al., 1981; Sato et al., 1985]. RA is an endogenous metabolite of vitamin A that exerts a wide range of biological effects on the growth and differentiation properties of normal, embryonic, and neoplastic cells [reviewed in Chambon et al., 1996]. RA promotes differentiation of various cell lines, including the murine P19 teratocarcinoma [Jones-Villeneuve et al., 1983] and the human HL-60 myeloblastic leukemia line [Breitman et al., 1980]. The actions of RA are mediated by nuclear receptors which belong to the steroid hormone receptor superfamily and act as ligand-inducible transcription factors [for reviews, see Chambon, 1996; Morriss-Kay and Sokolova, 1996]. The RA-mediated differentiation of P19 teratocarcinoma cells into a neuronal-like phenotype [Jones-Villeneuve et al., 1983] is accompanied by a rapid induction of *ALP* gene expression, increased levels of L/B/K ALP protein, and induced L/B/K isozyme activity in cell extracts determined at alkaline pH [Scheibe et al., 1991]. ALP activity is also induced by RA in the HL-60 leukemic cell line [Wei et al., 1992], which differentiates into granulocyte-like cells after exposure to RA [Breitman et al., 1981].

The physiological function of ALP as ecto-phosphatase remains controversial, mainly because early studies with purified ALP describe the enzyme as exhibiting a nonphysiological alkaline pH optimum [McComb et al., 1979]. Several other reports have demonstrated that the pH optimum for purified ALPs of various tissues and species largely depends on the kind of substrate and on substrate concentrations [Moss et al., 1961; Van Belle, 1976]. While the pH optimum was approximately 10.5, using the substrate *p*-nitrophenylphosphate (*p*NPP) at a concentration of 1≥mM [McComb et al., 1979], purified rat liver ALP had an optimal activity below pH 9.0 for several natural phosphomonoesters [Ohkubo et al., 1974]. In addition, a pH optimum of 7–8 was determined for purified and plasma membrane-bound human liver ALP [Chan and Stinson, 1986], as well as for purified bovine liver and calf intestine ALP [Swarup et al., 1981], using phosphohistones as substrates. A very broad pH optimum, extending between pH 6 and 10, has also been reported for purified human placental ALP with several phosphorylated proteins [Huang et al., 1976]. Furthermore, findings on purified human ALP isoenzymes and rat liver ALP demonstrated that the pH optimum for substrate hydrolysis decreased with decreasing concentration of the substrate and optimal activity at physiological pH was achieved with micromolar substrate concentrations [Moss et al., 1961; Sarrouilhe et al., 1993; Van Belle, 1976].

Plasma membranes of several cell types were found to express ecto-protein kinase activities involved in regulating cell-cell interactions, receptor functions, ion-channel activity, and association of extracellular proteins with cell surfaces [reviewed in Ehrlich et al., 1990; El-Moatassim et al., 1992]. It has been suggested that both protein kinases and phosphatases are required for reversible control of extracellular phosphorylation processes, acting together in a manner similar to that in which they control cytoplasmic phosphorylation/dephosphorylation systems [Hunter, 1995]. Ecto-protein kinase activities have also been detected on intact HL-60 myeloblastic leukemia [Paas and Fishelson, 1995] and differentiating neuronal cells [Pawlowska et al., 1993], and both cell types can release intracellular ATP required for efficient functioning of ecto-kinase/phosphatase systems [Buell et al., 1996]. Dephosphorylation of phosphoproteins of human plasma membranes by endogenous purified liver ALP has been described previously [Chan and Stinson, 1986]. However, investigations using the purified enzyme, fractions of plasma membrane vesicles, or cellular extracts give only limited insight into the enzyme functions in vivo.

In this study, we investigated ecto-ALP activity on the external surface of intact P19 and HL-60 cells and present evidence for its functional hydrolytic activity at pH 7.4. We report

the hydrolysis of *p*NPP at physiological pH and micromolar substrate concentrations. To distinguish ecto-ALP activity from other ecto-phosphatase activities, we used levamisole, which specifically inhibits activity of the L/B/K ALP isozyme at physiological pH in a stereospecific manner [Cyboron et al., 1982]. Levamisole was found to be a noncompetitive inhibitor of the hydrolytic activity on both cell types. Ecto-ALP activity was induced after treatment of cells with RA. Extracellular dephosphorylation of an autophosphorylated 98-kDa phosphoprotein on the surface of P19 cells increased by RA. Furthermore, addition of phosphatase inhibitors orthovanadate or levamisole blocked dephosphorylation of plasma membrane phosphoproteins, whereas addition of purified ALP increased dephosphorylation of the membrane proteins. Together, our data clearly support the argument that ALP functions in vivo as an ecto-phosphatase at physiological pH. Thus, P19 and HL-60 cells provide the opportunity to investigate the ecto-enzyme functions during neuronal and hematopoietic differentiation and development under physiological conditions.

## MATERIALS AND METHODS

### Materials

All-*trans*-RA, calf liver ALP, *p*NPP, levamisole, orthovanadate, penicillin-G, streptomycin, and trypsin were from Sigma-Aldrich (Deisenhofen, Germany). Malachite green was from Merck (Darmstadt, Germany). Bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) was purchased from Boehringer (Mannheim, Germany). All other products were analytical grade reagents from Sigma-Aldrich or Fluka AG (Neu-Ulm, Germany). [$\gamma$-$^{32}$P]ATP (3,000 Ci/mmol) and Rainbow™ protein molecular-weight markers were from Amersham (Braunschweig, Germany). $\alpha$-Minimal essential medium ($\alpha$-MEM), RPMI, and newborn calf serum were from Gibco (Karlsruhe, Germany). Bacteriological-grade Petri dishes were obtained from Greiner (Nuertingen, Germany), and tissue-culture dishes and flasks were from Nunc (Roskilde, Denmark).

### Cells and Culture Conditions

The mouse teratocarcinoma line P19 [Jones-Villeneuve et al., 1983; McBurney and Rogers, 1982] was grown in ($\alpha$-MEM) supplemented with 2.5% fetal calf serum (FCS) and 7.5% newborn calf serum. Human HL-60 myeloblastic cells were grown in liquid suspension in RPMI supplemented with 5% fetal bovine serum. Cells were maintained at 37°C in a 5% $CO_2$ atmosphere. All culture media contained penicillin-G (100 U/ml) and streptomycin (100 µg/ml) and were replaced after 2 days.

Cells were grown in the presence or absence of all-*trans*-RA at concentrations indicated in the figure legends. Differentiation of P19 cells was induced as follows. Cells were treated with phosphate-buffered saline (PBS) containing 0.125% (w/v) trypsin for 15 min. Cell suspension was pelleted (5 min at 1,000*g*), resuspended in medium with or without 1 µM RA and plated at a concentration of $10^6$ cells/ml into bacteriological-grade Petri dishes, where they aggregated spontaneously. The medium was replaced after 2 days, and the aggregates were transferred to tissue-culture dishes (60 mm), where they grew in monolayers. HL-60 cells were pelleted (5 min at 1,000*g*), resuspended in medium with or without 1 µM RA, and seeded at a concentration of $10^6$ cells/ml into tissue-culture flasks, where they grew in suspension.

For extracellular dephosphorylation studies cells ($10^6$ cells/ml) were plated in individual wells of 96-well plates and grown in the presence or absence of 1 µM RA for 48 h under culture conditions described above. Both before and after the phosphorylation/dephosphorylation assay, microscopic examinations were performed and verified that the morphology did not change during the assay.

### Phosphohydrolase Activity Assay for Ecto-ALP

Phosphohydrolase activity of ecto-ALP on the surface of intact cells was determined by a method described by Van Veldhoven and Mannaerts [1987]. Briefly, the procedure is based on the complex formation of malachite green with phosphomolybdate under acidic conditions, designed to measure inorganic orthophosphate ($P_i$) liberated from *p*NPP in a nanomolar range. The color developed during the first 20 min after the addition of malachite green, remaining stable for at least 24 h [Van Veldhoven and Mannaerts, 1987]. In the absence of $P_i$, the green color of the dye gradually disappeared, and the solution became yellow. The addition of polyvinyl alcohol (PVA) is required to stabilize the dye-phosphomolybdate complex. The assay was performed on the cell layers in culture

dishes in a humidified incubator in the absence of $CO_2$ at 37°C. The medium was removed, and cells were washed three times with pre-warmed reaction buffer A (135 mM NaCl, 4 mM KCl, 1 mM $CaCl_2$, 20 mM Hepes, and 5 mM glucose, adjusted to pH 7.4); 4 ml of buffer A was supplemented with 1 mM $MgCl_2$ and 250 µM *p*NPP as substrate. After 30-min intervals, 100 µl of the supernatant solution was transferred to plastic tubes, and 200 µl of reagent B (1.75% (w/v) ammonium heptamolybdate × $4H_2O$ in 6.3N $H_2SO_4$) was added. After 10 min at 22°C, 200 µl of reagent C (0.035%, w/v, malachite green, 0.35%, w/v, PVA in $H_2O$) was added and mixed again. The absorbance at 610 nm was determined 30 min later in semimicrocuvettes (1-cm lightpath) in a spectrophotometer. Most measurements were performed at pH 7.4. ALP activity was also determined both in the presence and in the absence of 1 mM levamisole. In some experiments, buffer A was supplemented with various amounts (5–15 mM) of Hepes or Tricine, or both, to obtain different pH values over the range of 6.7–8.5 [Eagle, 1977]. One unit (1 U) of enzyme activity was defined as the amount of enzyme needed to release 1 nmol *P*i from *p*NPP/min/ml. Results are expressed as U/mg of cell protein. Protein was determined according to the method of Oyama and Eagle [1978].

Bacterial PI-PLC was used to release ecto-ALP from the plasma membrane and to determine ecto-ALP activity in the incubation medium. Cells grown on 96-well plates were washed twice with NaCl-free buffer A and were then incubated in the same buffer without NaCl for successive periods of 30 min at 37°C, pH 7.4, in the presence or absence of 0.2 U/ml PI-PLC, whose activity was inhibited by NaCl [Sundler et al., 1978]. At the end of each incubation period, buffer was collected and 135 mM NaCl and 1 mM $MgCl_2$ was added before determination of *p*NPP hydrolysis and *p*NPPase activity released into the buffer by the method described above.

### Extracellular Phosphorylation Studies

Experiments involving extracellular phosphorylation of cell surface proteins were performed with intact cells. Cells were grown in 96-well plates in the presence and absence of 1 µM RA for 48 h and washed three times in reaction buffer B (150 mM NaCl, 5.5 mM KCl, 3.8 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 5 mM glucose, 20 mM Hepes, pH 7.4). Autophosphorylation of plasma membrane proteins by extracellular protein kinase activity was started by addition of 5 µCi/100 µl of [$\gamma$-$^{32}$P]ATP (3,000 Ci/mmol in 16 nM ATP, final concentration) and cells were incubated for 0–30 min at 37°C. Reactions that tested the effects of trypsin (0.01%, w/v) or apyrase (5 U/ml) were initiated 10 min before the addition of [$\gamma$-$^{32}$P]ATP. Levamisole (1 mM) was added to some samples to inhibit ecto-ALP activity. Cells were then homogenized; trichloroacetic acid (TCA) was added to a final concentration of 15% (w/v). The precipitate was filtered onto glass fiber filters (Whatman, Kent, England). Filters were washed twice with PBS, dried at 60°C, and transferred to vials, to which 3 ml scintillation fluid was added. Filters were assayed for radioactivity in a Berthold CS 5000 liquid scintillation counter (Berthold GmbH, Bad Wildbad, Germany) using Quickzint 2000 as the scintillant and [$^{32}$P]phosphate incorporated in membrane proteins was determined.

### Extracellular Dephosphorylation Studies

Cells were grown in the presence or absence of RA for 48 h and plasma membrane proteins were autophosphorylated in reaction buffer B, using [$\gamma$-$^{32}$P]ATP as described above, but at a short reaction period of 3 min at 37°C. In addition, orthovanadate (1 mM) was present in buffer B to inhibit phosphatase activities during autophosphorylation reaction and incorporation of [$^{32}$P]phosphate into cell surface proteins. After the 3-min incubation period at 37°C, cells were washed three times with buffer B at 4°C. Dephosphorylation of $^{32}$P-labeled plasma membrane proteins was initiated by the addition of 1 mM (R)-(−)-epinephrine, which complexes orthovanadate; P19 and HL-60 cells were incubated at 37°C for an additional 30 min and 40 min, respectively. In some cell cultures, phosphorylation buffer B was supplemented with 1 mM levamisole or calf liver ALP (1 U/ml) before the addition of 1 mM (R)-(−)-epinephrine. Other cultures were treated with trypsin (0.01%, w/v) for 10 min before or after the addition of (R)-(−)-epinephrine and incubation for 3 min. At the end of each incubation, cells were washed three times with buffer B. Extracellular dephosphorylation of cell surface proteins was determined in HL-60 cultures by TCA precipitation as described above. P19 cells were lysed in 200 µl of 62 mM Tris/HCl, pH 7.0, containing 10% (w/v) glycerol, 5% (v/v) β-mercaptoethanol, and 2.3% (w/v) sodium dodecyl sulfate (SDS). Analysis of

dephosphorylated proteins was performed by SDS-polyacrylamide gel electrophoresis (PAGE) on a 12% polyacrylamide gel as described [Laemmli, 1970], and incorporation of radioactive phosphate was determined by autoradiography and scanning densitometry. Experimental data were expressed as integrated values of band densities (arbitrary units). Background was subtracted by using values from scanning of a lane not loaded with sample. The range of band densities was within the linear range of the XAR films (Kodak, Sigma-Aldrich, Deisenhofen, Germany).

### Cell Viability and Permeability Studies

Cells were grown on 96-well plates for 24 h. Cell viability under phosphorylation/dephosphorylation conditions used above was tested as follows. Cultures were exposed to 16 nM ATP or 1 mM orthovanadate for 3 min at 37°C, or to 1 mM levamisole or 1 mM (R)-(−)-epinephrine for 10 min at 37°C in reaction buffer B. Reaction buffer B was then removed and replaced with 100 µl of detergent-containing lysing solution, providing a uniform suspension of single, intact viable nuclei [Soto and Sonnenschein, 1985]. Nuclei were quantified by counting in a hemacytometer. Broken or damaged nuclei were not included in the counts.

To determine cell permeability under phosphorylation/dephosphorylation conditions, cells were grown on 96-well plates for 24 h. Medium was replaced with 200 µl pre-warmed reaction buffer B and cells were incubated at 37°C for 3 or 10 min with either [$\gamma$-$^{32}$P]ATP, levamisole, orthovanadate, or (R)-(−)-epinephrine at the concentrations used above, or 0.025% Triton. Cells were then incubated for 30 s with 5 µg/ml ethidium bromide, which instantly stains permeable cells. Cells were washed and counted with a fluorescence microscope in 10 different fields from each coverslip.

## RESULTS

### Levamisole-Sensitive Ecto-Phosphohydrolase Activity on Intact P19 and HL-60 Cells

In order to investigate the phosphohydrolase activity on the external surface of P19 teratocarcinoma and HL-60 myeloblastic cells, substrate hydrolysis was determined at physiological conditions (37°C, pH 7.4). We used *p*NPP as substrate for the in vivo studies because it does not penetrate the plasma membrane [DePierre and Karnovsky, 1974]. Replicate cultures were grown for 3 days after the cell passage, and cells were washed with a reaction buffer mimicking a physiological extracellular environment (see Materials and Methods). Reaction buffer containing substrate was added, and cells were submitted to eight successive incubations of 30 min. Cells were assayed for extracellular substrate hydrolysis while growing on culture dishes or in flasks. The reaction conditions did not affect cellular survival, permeability (Tables II, III), and cell morphology determined by microscope examination (data not shown) for the next 3 days.

The assay is based on the hydrolysis of phosphate esters by ecto-ALP, leading to an increase in $P_i$ and to a complex formation of malachite green with phosphomolybdate. As shown in Figure 1, the rate of hydrolysis on the external surface of P19 cells determined at each of the eight incubation times was about 10 U/mg cell protein and remained constant over the period of 240 min. The addition of levamisole (1 mM), a specific inhibitor of L/B/K ALP activity [Cyboron et al., 1982; Van Belle, 1976], inhibited ecto-phosphohydrolase activity by about 90% (Fig. 1). When levamisole was removed after a 120-min incubation period, ecto-phosphohydrolase activity was restored within 60 min and reached values of untreated controls. Levamisole-sensitive phosphohydrolase activity was also detected on intact HL-60 cells with *p*NPP as the substrate (Table I). The rate of hydrolysis on the external surface of HL-60 cells was about 8 U/mg cell protein. After addition of the specific inhibitor of ALP, we measured an about 95% inhibition of ecto-phosphohydrolase activity (Table I) at physiological pH (7.4), using *p*NPP. As for P19 cells, the inhibitory effect in HL-60 cells was reversed within 60 min after removal of the inhibitor. The results indicate, that under physiological conditions, phosphohydrolase activity of ALP is expressed on the cell surface of P19 and HL-60 cells.

### RA-Induced Levamisole-Sensitive Ecto-Phosphohydrolase Activity at Physiological pH

Previously, ALP activity has been determined in cell extracts of the P19 and HL-60 line at alkaline pH, using *p*NPP as the substrate, and found to be induced after treatment with RA for 48 h [Scheibe et al., 1991; Wei et al.,

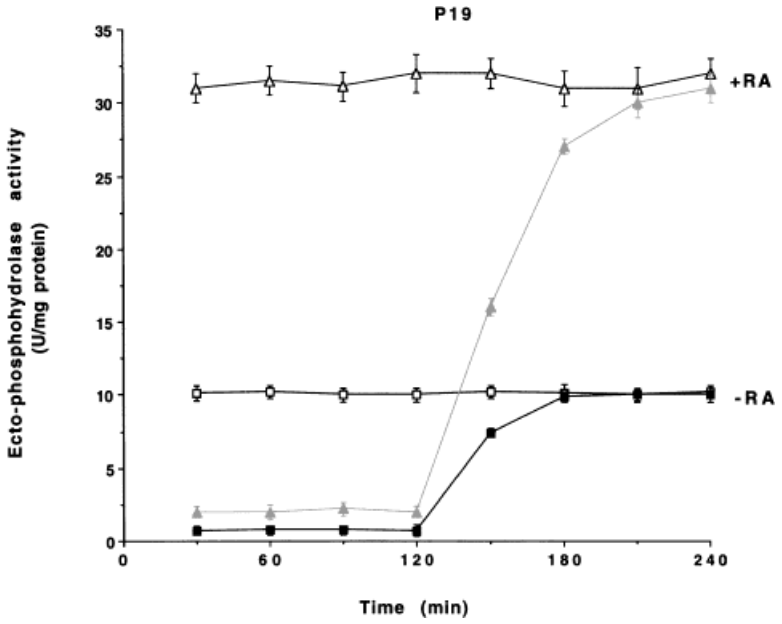


**Fig. 1.** Levamisole-sensitive phosphohydrolase activity on intact P19 cells in the presence and absence of retinoic acid (RA). Cells were grown in the presence (triangle) or absence (square) of 1 μM RA for 2 days. Intact cells were then incubated with reaction buffer containing 250 μM *p*-nitrophenylphosphate (*p*NPP) at pH 7.4, 37°C, and ecto-*p*NPP hydrolase activity was measured for eight successive 30-min incubations. At the end of each incubation, the supernatant solution was analyzed for the release of $P_i$ and for subsequent complex formation of malachite green with phosphomolybdate as described under Materials and Methods. Cultures were incubated in the presence (solid symbols) or absence (open symbols) of 1 mM levamisole. Levamisole was present in the reaction mixture for the first four incubation periods (120 min) and then removed from the assay. Values represent the means ± SD of three independent experiments in triplicate determinations. Results are expressed as units per mg of cell protein.

1992]. To investigate the effect of RA on ecto-ALP activity on the external surface of P19 and HL-60 cells at physiological conditions (pH 7.4), substrate hydrolysis was determined with *p*NPP. Cells were grown for 2 days in the presence of RA (1 μM); the rate of hydrolysis was then measured on intact cells at eight successive incubations of 30 min. Ecto-phosphohydrolase activities in P19 cells at each of the eight incubation times were about 32 U/mg cell protein (Fig. 1). In HL-60 cells, ecto-phosphohydrolase activities were about 25 U/mg cell protein (Table I). Values remained constant over the period of 240 min. Thus, RA-treated P19 and HL-60 cells expressed an approximately threefold increase in ecto-phosphohydrolase activity at physiological pH, compared with untreated controls. When levamisole (1 mM) was present in the reaction buffer for the first 120 min, RA-induced hydrolysis of *p*NPP was inhibited by more than 90% at pH 7.4 in both cell types (Fig. 1 and Table I). Together, the results demonstrate that levamisole strongly inhibited RA-induced ecto-*p*NPP hydrolase activity in differentiating P19 and HL-60 cells.

**TABLE I. Levamisole-Sensitive Ecto-Phosphohydrolase Activity on Intact HL-60 Cells***

| | Ecto-phosphohydrolase activity (U/mg protein) | |
|---|---|---|
| Time (min) | −RA | +RA |
| −Levamisole | | |
| 30 | 8.15 ± 0.25 | 25.15 ± 2.0 |
| 60 | 8.04 ± 0.21 | 25.72 ± 1.9 |
| 120 | 8.09 ± 0.19 | 25.02 ± 1.9 |
| 180 | 8.01 ± 0.20 | 25.26 ± 2.1 |
| 240 | 8.11 ± 0.23 | 25.18 ± 2.2 |
| +Levamisole | | |
| 30 | 0.45 ± 0.07 | 0.21 ± 0.1 |
| 60 | 0.48 ± 0.05 | 0.28 ± 0.2 |
| 120 | 0.47 ± 0.06 | 0.25 ± 0.1 |
| 180 | 8.02 ± 0.21 | 22.03 ± 2.0 |
| 240 | 8.10 ± 0.23 | 25.11 ± 2.2 |

*Cells were incubated with assay buffer, pH 7.4, containing *p*NPP. Ecto-phosphohydrolase activity was determined for eight successive incubations of 30 min as described for Fig. 1A; results are shown at the time points indicated. Levamisole was present in the reaction mixture for 120 min and then removed from the assay. Values represent the means ± SD of three independent experiments in triplicate determinations.

### Release of Levamisole-Sensitive *p*NPP Hydrolase Activity by PI-PLC

The membrane-impermeable PI-PLC selectively removes glycophospholipid-linked proteins, including the ALP from membranes [Low and Finean, 1977]. To determine further the contribution of the glycosylphosphatidylinositol-anchored ALP to the ecto-phosphohydrolase activity, we incubated intact cultures with reaction buffer containing the substrate *p*NPP and measured hydrolysis of *p*NPP in the reaction buffer before and after treatment with PI-PLC (Fig. 2). Cells were first incubated for two 30-min incubation periods in the absence of PI-PLC, during which no *p*NPP hydrolase activity was detected. Addition of PI-PLC to cultured cells resulted in a release of *p*NPPase activity into the reaction buffer. In the presence of levamisole, the released *p*NPP hydrolase activity was totally inhibited. Protein content and cell detachment were not modified during PI-PLC treatment (data not shown). The results suggest that the PI-PLC-induced release of levamisole-sensitive *p*NPPase activity was due to the release of ecto-ALP into the reaction buffer. Thus, the levamisole-sensitive *p*NPP hydrolase activity measured on the surface of intact P19 and HL-60 cells is now being referred to as ecto-ALP activity.

### pH Dependence of the Ecto-ALP Activity

To determine the effect of pH for the levamisole-sensitive ecto-ALP activity, substrate hydrolysis on intact cultured cells was measured as a function of pH. The reaction buffer was supplemented with various concentrations of organic buffers permitting the pH to vary between 6.7 and 8.5 [Eagle, 1977]. We determined ecto-ALP activity in P19 and HL-60 cells using *p*NPP at a final concentration of 250 µM. The results shown in Figure 3A demonstrate that at pH 6.7–7.4, hydrolysis of *p*NPP increased in both cell types and then nearly plateaued pH 7.4–8.5. The values of activity obtained with *p*NPP at pH 6.8 and 7.4 increased in P19 cells by 2.0-fold and in HL-60 cells by 2.4-fold. The addition of levamisole inhibited ecto-ALP activity and inhibition was independent of the buffer pH. These results demonstrate that ecto-ALP expressed on the surface of intact P19 and HL-60 cells was active at physiological pH range.

The pH optimum for purified ALPs has been reported to depend on substrate concentrations and to decrease with decreasing concentrations of the substrate [Sarrouilhe et al., 1993; Van Belle, 1976]. Figure 3B shows that ecto-ALP activity at the physiological pH of 7.4 reached

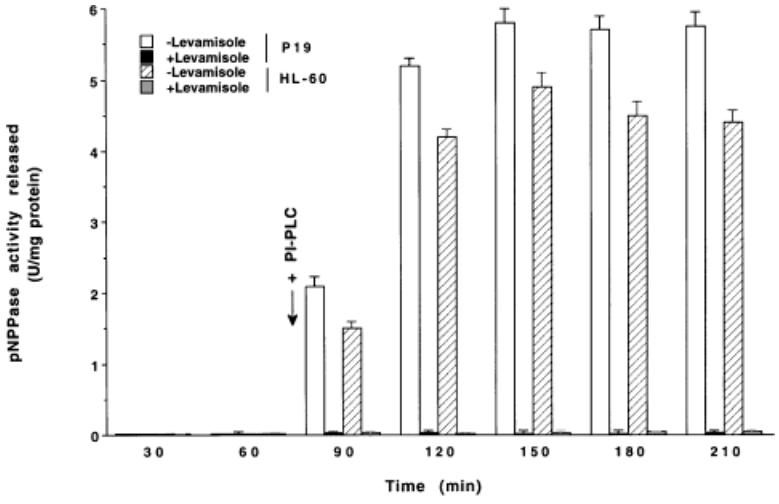


**Fig. 2.** Effect of phosphatidylinositol-specific phospholipase C (PI-PLC) on the release of levamisole-sensitive *p*NPP hydrolase activity. Bacterial PI-PLC was used to release ecto-alkaline phosphatase (ALP) from the plasma membrane of intact P19 and HL-60 cells and to determine levamisole-sensitive *p*NPP hydrolase activity in the incubation medium. Cells were washed twice with NaCl-free buffer A and were incubated in the same buffer without NaCl for successive periods of 30 min at 37°C, pH 7.4. The first two incubation periods were performed in the absence of PI-PLC, and 0.2 U/ml PI-PLC was added after 60 min. At the end of each incubation period, buffer was collected and 135 mM NaCl and 1 mM $MgCl_2$ were added before determination of *p*NPP hydrolase activity released into the buffer in the presence and absence of 1 mM levamisole, as described under Materials and Methods. Values represent the means ±SD of three independent experiments.

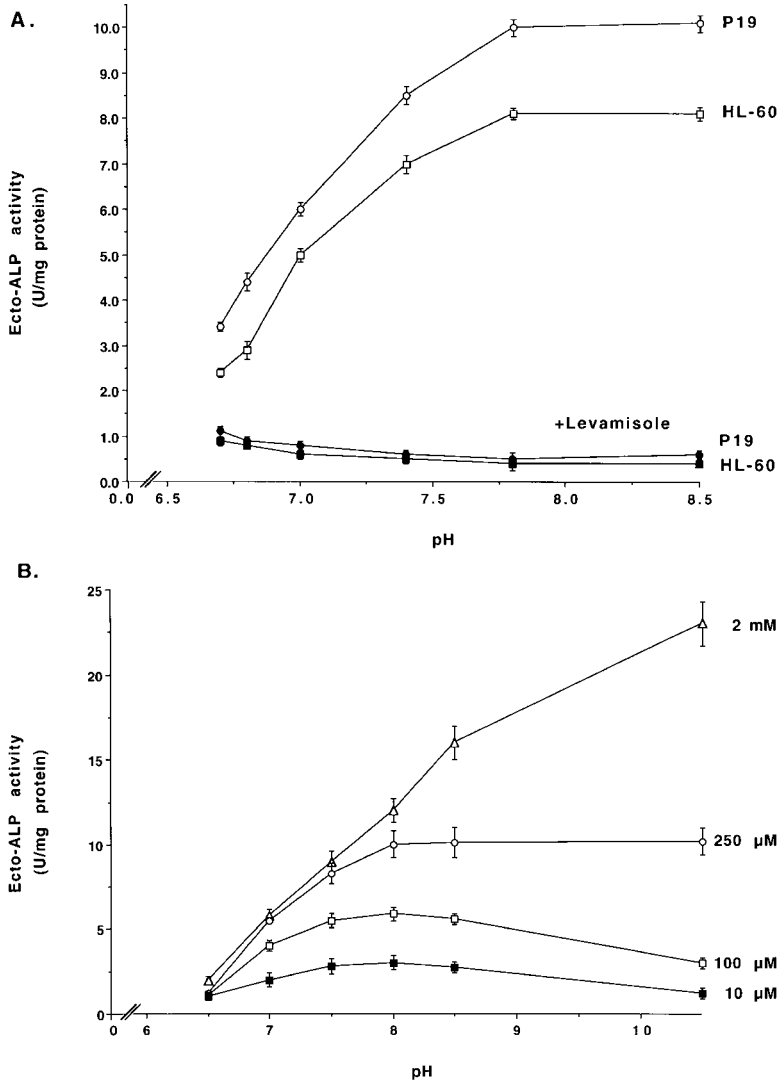


**Fig. 3.** Effect of pH on ecto-alkaline phosphatase (ALP) activity. **A:** P19 (circle) and HL-60 cells (square) were incubated with reaction buffer containing 250 µM *p*NPP. The buffer was 135 mM NaCl, 4 mM KCl, 1 mM $CaCl_2$, 5 mM glucose, and organic buffer additives Hepes (10–20 mM) and Tricine (2.5–15 mM) to obtain various pH over the range of 6.7 and 8.5. Incubation of intact cultured cells was performed for 60 min in the presence (solid symbols) or absence (open symbols) of 1 mM levamisole. **B:** Interdependence of pH and substrate concentration. Cells were incubated with reaction buffer as described in A, containing various concentrations of *p*NPP (0.01–2.0 mM). Incubation of intact cultured cells was performed for 60 min; hydrolysis of *p*NPP was determined by the release of $P_i$ and subsequent complex formation of malachite green with phosphomolybdate as described under Materials and Methods. Results are expressed as means ± SD of three independent experiments in duplicate determinations.

about 80% of the activity at pH 10.5, using a substrate concentration of 250 µM *p*NPP. When we lowered the substrate concentration to 100 µM or 10 µM the pH optimum of ecto-ALP in P19 (Fig. 3B) and HL-60 cells (data not shown) was found to decrease. Although hydrolysis of *p*NPP at 10 µM and 100 µM was limited, maximal enzyme activity was maximal between pH 7.4 and 8.5.

### Kinetic and Inhibition Parameters of Ecto-ALP

We next determined the kinetic and inhibition parameters of ecto-ALP at physiological pH, using *p*NPP as the substrate and levamisole as the inhibitor. The assays were performed with increasing concentrations of *p*NPP (0.015–1 mM) and levamisole (0.01–0.5 mM). Figure 4A summarizes kinetic analysis with

*p*NPP at pH 7.4. The value of $K_m$ using *p*NPP was 120 μM with intact P19 and HL-60 cells. Values for $V_{max}$ in P19 and HL-60 cells were 3.3 nmol/min/mg in the absence of RA. When cells were treated with RA for 48 h, values for $V_{max}$ increased to 7.3. As shown in Figure 4B, levamisole inhibited hydrolysis of *p*NPP by ecto-ALP in a dose-dependent manner. The intersection between $V_o/V_{app}$ versus levamisole concentration indicated an apparent $K_i$ of about 57 μM in P19 and apparent $K_i$ of about 50 μM in HL-60 cells. With the substrate *p*NPP at pH 7.4, levamisole proved to be a noncompetitive inhibitor of ecto-ALP.

### Ecto-ALP Activity Dephosphorylated a 98-kDa Cell Surface Protein in P19 Cells

In characterizing the ecto-ALP activity on the external cell surface, we determined dephosphorylation of phosphoproteins in the plasma

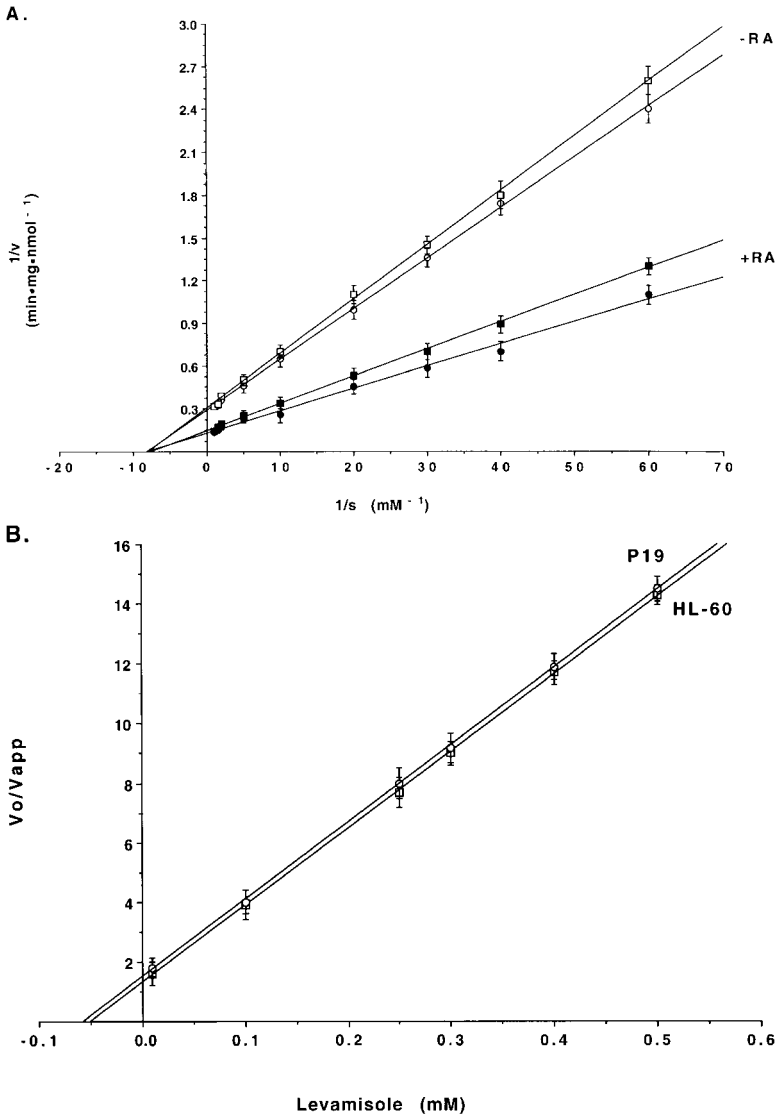


**Fig. 4.** Kinetic and inhibition parameters of ecto-alkaline phosphatase (ALP). **A:** Kinetic analysis at physiological pH. P19 (circle) and HL-60 (square) cells were grown in the presence (closed symbols) or absence (open symbols) of 1 μM RA for 48 h. Cells were washed and incubated with reaction buffer containing increasing concentrations of *p*-nitrophenylphosphate (*p*NPP) (0.015–1 mM), and ecto-ALP activity was measured at pH 7.4. Kinetic parameters were determined by graphic analysis. **B:** Inhibition of *p*NPP hydrolysis by levamisole. Cells were incubated with reaction buffer containing 250 μM *p*NPP and increasing concentrations of levamisole (0.01–0.5 mM). Ecto-ALP activity on intact P19 (circle) and HL-60 cells (square) was measured at pH 7.4. $V_o$ is $V_{max}$ in the absence of inhibitor, $V_{app}$ is $V_{max}$ in the presence of inhibitor. Bars represent the standard error of the parameter, as determined by Cleland's Hyper-fit program [Cleland, 1979]. Lines are drawn according to a linear least-squares fit.

membrane. Cells were grown in 96-well plates and first autophosphorylated by incubation with [γ-$^{32}$P]ATP, which does not penetrate cells [Ehrlich et al., 1990; Gordon, 1986]. Ecto-protein kinase activity was detected on intact P19 and HL-60 cells resulting in incorporation of [$^{32}$P]phosphate into cell surface proteins as early as 1 min and was enhanced during the 30-min incubation period (Fig. 5). The phosphorylation of proteins in P19 cells increased in the presence of levamisole and was prevented by adding apyrase, an ATP-hydrolyzing enzyme [Ehrlich et al., 1990], or trypsin to the reaction buffer 10 min before the incubation with [γ-$^{32}$P]ATP.

To determine possible effects of RA-induced ecto-ALP activity on the extracellular dephosphorylation of membrane proteins, P19 cells were preincubated with RA for 24 h and 48 h before initiation of autophosphorylation by a 3-min reaction with [γ-$^{32}$P]ATP. As shown in Figure 6A, only a few proteins were labeled after the short incubation period with radiolabeled ATP; three bands with apparent molecular weights of 23, 66, and 98 kDa were most prominent. Figure 6B depicts the quantitative results of the phosphorylation/dephosphorylation state of the 98-kDa protein. RA treatment selectively decreased abundance of the 98 kDa band (Fig. 6A, lanes 4 and 5) compared with untreated P19 controls (lane 1). Phosphorylation was about 55% and 96% reduced after treatment with RA for 24 h and 48 h, respectively (Fig. 6B). To ascertain that the phosphoproteins were cell surface proteins, P19 cells were exposed for 15 min to low concentrations of trypsin (0.01%, w/v) before (lane 2) or after (lane 3) the incubation with [γ-$^{32}$P]ATP. Both treatments with trypsin markedly inhibited phosphorylation of the 98-, 66-, and 23-kDa proteins. The data strongly suggest that all three proteins are phosphorylated extracellularly because trypsin, which is unable to penetrate cells, is nevertheless capable of inhibiting such a phosphorylation. In addition, no visible phosphorylation of the 98-, 66-, and 23-kDa proteins was detected by incubating intact cells under the same experimental conditions as above, but in the presence of [$^{32}$P]orthophosphate for 10 min (data not shown). This finding indicates that phosphorylation of these proteins was not achieved intracellularly after breakdown of the exogenous [γ-$^{32}$P]ATP and subsequent uptake and incorporation of the phosphate onto intracellular ATP.

To further investigate plasma membrane protein dephosphorylation by ecto-ALP, it was necessary to assess separately the ecto-kinase and

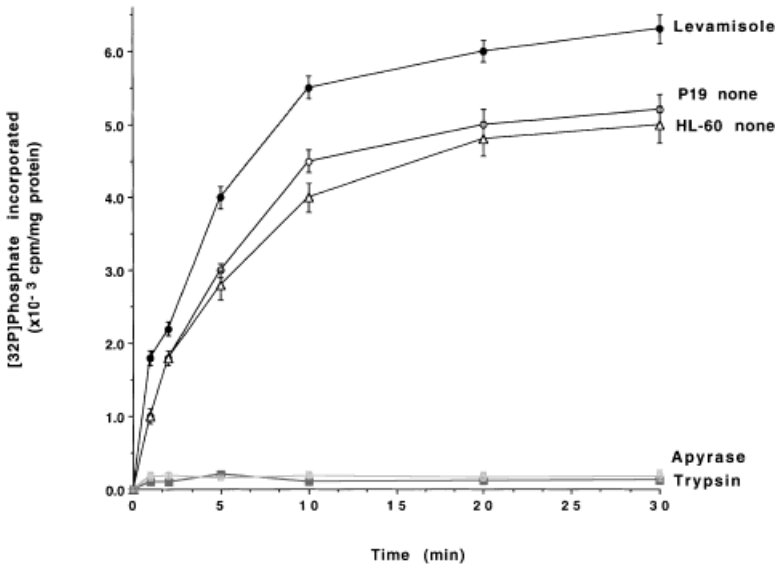


**Fig. 5.** Extracellular phosphorylation of plasma membrane proteins. Intact P19 and HL-60 cells were incubated in 96-well plates with phosphorylation buffer containing 5 μCi/100 μl of [γ-$^{32}$P]ATP for 0–30 min at 37°C. Lysis of cells and determination of incorporated [$^{32}$P]phosphate were performed as described under Materials and Methods. Additions were none for both cell types (○, △), and for P19 cells 1 mM levamisole (●), 0.01% (w/v) trypsin (■), or 5 U/ml apyrase (□) were added 10 min before the addition of [γ-$^{32}$P]ATP. Results are means ± SD of three independent experiments.

ecto-phosphatase activities. Therefore, we studied the effect of orthovanadate, levamisole, and purified calf liver ALP on the dephosphorylation state of membrane phosphoproteins in HL-60 cells. The presence of orthovanadate, an inhibitor of all phosphatases, including the ALP [Seargeant and Stinson, 1979a], during the phosphorylation reaction strongly reduced dephosphorylation of the 98 kDa protein in cultures grown in the presence of RA for 48 h and enhanced abundance of the radiolabeled protein band to the level detected in untreated control cultures (Fig. 6A; cf. lanes 1 and 6). The inhibitory effect of orthovanadate on protein phosphatase activities was reversed by further addition of (R)-(−)-epinephrine (lane 7), due to the chelation of the inhibitor by the amine [Seargeant and Stinson, 1979a]. The phosphorylated 98 kDa plasma membrane protein was not dephosphorylated until (R)-(−)-epinephrine was added. Furthermore, this dephosphorylation could be inhibited by levamisole (lane 8) or accelerated by the addition of purified ALP (lane 9); the addition of calf liver ALP led to an approximately 98% decrease in phosphorylation (Fig. 6B). The results demonstrate that RA increased dephosphorylation and suggest that RA did not reduce synthesis of the 98-kDa membrane protein. Together, the data show the presence of an RA-induced and levamisole-sensitive ecto-ALP activity affecting the phosphorylation state of the 98-kDa plasma membrane protein.

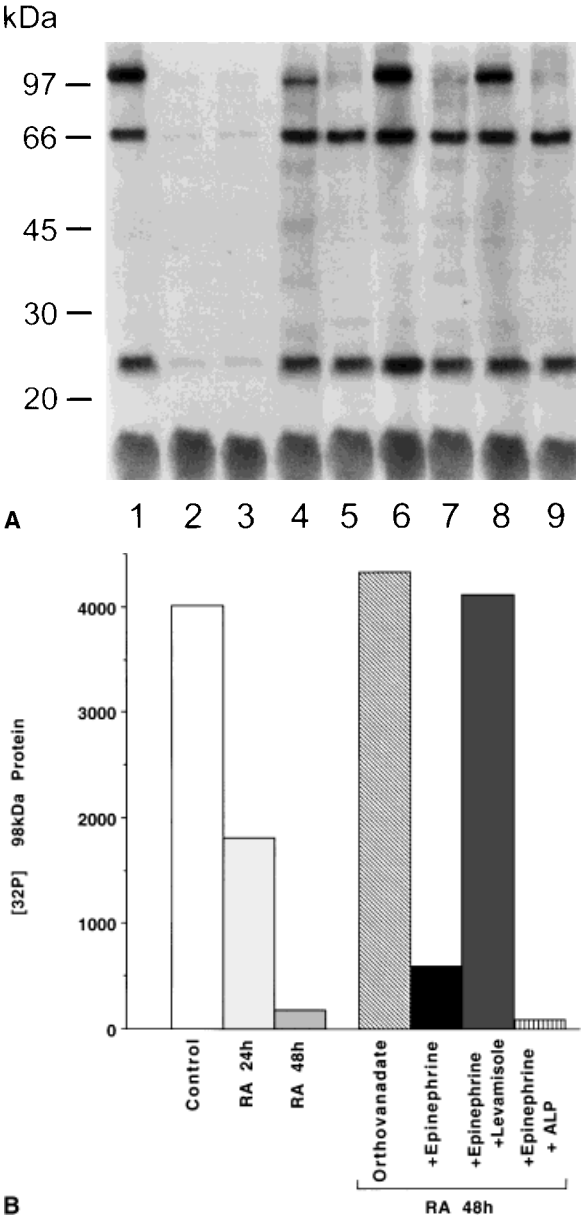


**Fig. 6.** Extracellular dephosphorylation of a [$^{32}$P]phosphate-labeled plasma membrane protein in P19 cells. **A:** Cells were grown on 96-well plates in the absence (**lanes 1–3**) or presence of 1 μM RA for 24 h (**lane 4**) or 48 h (**lanes 5–9**). Medium was replaced by phosphorylation buffer and cells were incubated with 5 μCi/100 μl of [γ-$^{32}$P]ATP for 3 min at 37°C, as described under Materials and Methods. Some control cultures were also treated with trypsin (0.01%) for 15 min before (**lane 2**) or after (**lane 3**) the incubation with [γ-$^{32}$P]ATP. After exposure to RA for 48 h, some cells were phosphorylated in the presence of 1 mM orthovanadate (**lanes 6–9**). Cells were washed, and dephosphorylation was initiated by further incubation for 30 min with 1 mM (R)-(−)-epinephrine (**lane 7**), (R)-(−)-epinephrine plus 1 mM levamisole (**lane 8**), and (R)-(−)-epinephrine plus calf liver ALP (1 U/ml) (**lane 9**). Cell lysis and SDS-PAGE were performed as described under Materials and Methods. Results of the phosphorylation/dephosphorylation reactions were visualized by autoradiography. The position of the molecular weight standards are indicated on the left. Comparable results were obtained in three independent experiments. **B:** Quantification of [$^{32}$P]phosphate incorporation into the 98-kDa plasma membrane protein and dephosphorylation was performed by laser densitometric scanning of autoradiograms. Experimental data were expressed as integrated values of band densities (arbitrary units).

### Characterization of Ecto-Phosphatase Activity and Extracellular Dephosphorylation of HL-60 Cell Surface Proteins

Extracellular phosphorylation/dephosphorylation of surface proteins in HL-60 cultures was determined by a similar procedure. Ecto-protein kinase activities on HL-60 cells have already been described previously that were capable of phosphorylating exogenously added substrates such as phosvitin and poly(Glu4-Tyr) [Paas and Fishelson, 1995]. In the present study, HL-60 cells were incubated with

[γ-$^{32}$P]ATP and autophosphorylation by ecto-kinase activity was determined over a period of 0–30 min. For dephosphorylation studies, cells surface proteins were autophosphorylated at a short 3-min incubation period in the presence of orthovanadate under the above condition to inhibit phosphatase activities. After cells were lysed and proteins precipitated, the amount of $^{32}$P incorporated was determined. Figure 7 shows that because of the presence of orthovanadate phosphorylated HL-60 membrane proteins were not dephosphorylated until (R)-(−)-epinephrine was present in the assay. The dephosphorylating process was inhibited in the presence of (R)-(−)-epinephrine plus levamisole or enhanced with (R)-(−)-epinephrine plus purified calf liver ALP. The amount of $^{32}$P remaining after the addition of (R)-(−)-epinephrine and incubation for 30 min was reduced to about 20% in RA-treated (epinephrine$_{RA}$) and about 35% in control cultures (epinephrine$_{Con}$) compared with cultures in the absence of epinephrine (no epinephrine). The RA-induced increase of dephosphorylation of cell surface proteins was probably attributable to the higher ecto-ALP activity found on HL-60 plasma membranes.

Experiments were performed to control that cellular viability and permeability were not impaired under the conditions adopted for the phosphorylation/dephosphorylation studies. Cells were exposed to the phosphorylating/dephosphorylating conditions described above and assessed immediately or 3 days later for cellular survival (Tables II, III). The results demonstrate that the phosphorylation/dephosphorylation conditions per se did not modify cell viability measured by direct count of intact viable nuclei (Table II) and by immediate uptake of ethidium bromide (Table III). The number of fluorescence-stained cells was comparable in control and ATP-, orthovanadate-, and levamisole-treated samples but was found to be markedly augmented in the presence of known permeabilizing agents such as Triton.

## DISCUSSION

This study demonstrates that cultured P19 teratocarcinoma and HL-60 myeloblastic leukemia cells possess phosphatase activity capable of hydrolyzing *p*NPP under physiological pH conditions. The experimental evidence allowed us to characterize this activity as an ecto-phosphatase. The three main arguments in fa-

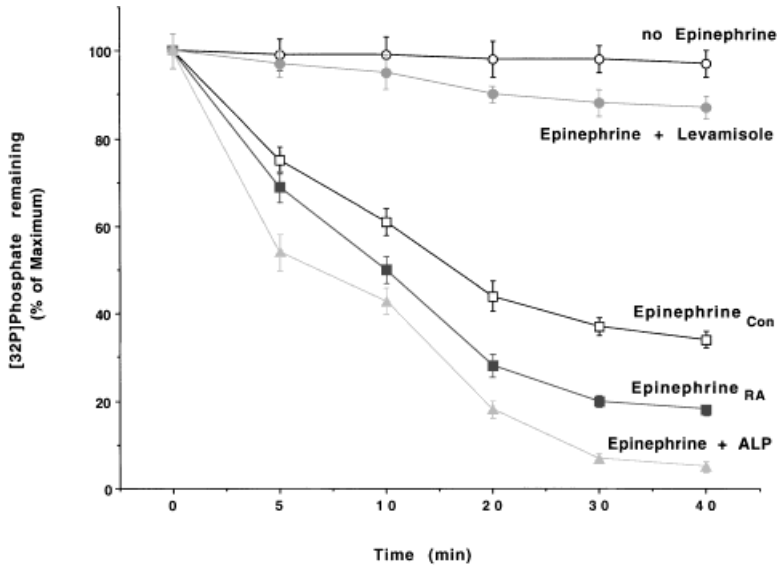


**Fig. 7.** Dephosphorylation of [$^{32}$P]phosphate-labeled plasma membranes in HL-60 cells. Intact cells were autophosphorylated in the presence of 1 mM orthovanadate with [γ-$^{32}$P]ATP for 3 min at 37°C as described under Materials and Methods. Cells were then washed, and further incubated for 40 min with no (R)-(−)-epinephrine, 3 mM (R)-(−)-epinephrine (epinephrine$_{Con}$), (R)-(−)-epinephrine + 1 mM levamisole, (R)-(−)-epinephrine + 1 U/ml calf liver ALP. In addition, cells grown in the presence of 1 μM RA for 48 h were phosphorylated in the presence of orthovanadate and incubated with (R)-(−)-epinephrine (epinephrine$_{RA}$) under the same conditions. The content of $^{32}$P remaining in plasma membranes was determined as described under Materials and Methods. Results are means ±SD of three independent experiments. Values are expressed as the percentage of $^{32}$P remaining compared with the maximum of $^{32}$P incorporation into plasma membranes in the absence of dephosphorylation (no epinephrine).

**TABLE II. Extracellular Phosphorylation/Dephosphorylation Conditions Did Not Affect Cell Viability***

| | | % of intact nuclei | | | | |
|---|---|---|---|---|---|---|
| Cells | Time | Control | ATP | Orthovanadate | Levamisole | Epinephrine |
| P19 | 0 h | 100 ± 12 | 98 ± 12 | 98 ± 14 | 99 ± 10 | 100 ± 12 |
| | 3 d | 100 ± 19 | 102 ± 13 | 97 ± 10 | 104 ± 11 | 98 ± 11 |
| HL-60 | 0 h | 100 ± 10 | 97 ± 14 | 98 ± 13 | 101 ± 12 | 103 ± 12 |
| | 3 d | 100 ± 13 | 103 ± 15 | 97 ± 16 | 98 ± 19 | 99 ± 13 |

*Cells were grown on 96-well plates for 24 h. Medium was replaced with reaction buffer and cells were incubated at 37°C for 3 min with or without 16 nM ATP or 1 mM orthovanadate, or at 37°C for 10 min with or without 1 mM levamisole or 1 mM (R)-(−)-epinephrine. Cells were assessed immediately (0 h) or 3 days later (3 d) for survival by direct count of intact nuclei as described under Materials and Methods. Measurements were performed in triplicate, and data are means ± SD values of three separate experiments. 100% of intact nuclei represent $1 \times 10^5$ viable cells determined at 0 h and $3 \times 10^5$ cells determined at 3 days in untreated control cultures.

**TABLE III. Extracellular Phosphorylation/Dephosphorylation Conditions Did Not Affect Cellular Permeability***

| | | % of cellular permeabilization | | | | | |
|---|---|---|---|---|---|---|---|
| Cells | Time | Control | ATP | Orthovanadate | Levamisole | Epinephrine | Triton |
| P19 | 0 h | 0.23 ± 0.02 | 0.18 ± 0.01 | 0.23 ± 0.02 | 0.22 ± 0.02 | 0.19 ± 0.02 | 4.13 ± 0.35 |
| | 3 d | 0.22 ± 0.01 | 0.21 ± 0.02 | 0.20 ± 0.03 | 0.24 ± 0.03 | 0.18 ± 0.03 | 4.51 ± 0.38 |
| HL-60 | 0 h | 0.22 ± 0.01 | 0.19 ± 0.01 | 0.21 ± 0.01 | 0.20 ± 0.02 | 0.18 ± 0.01 | 5.15 ± 0.48 |
| | 3 d | 0.21 ± 0.02 | 0.20 ± 0.02 | 0.18 ± 0.02 | 0.22 ± 0.01 | 0.19 ± 0.02 | 5.42 ± 0.49 |

*Cells were grown on 96-well plates for 24 h. Medium was replaced with reaction buffer, and cells were incubated at 37°C for 3 min with or without 16 nM ATP or 1 mM orthovanadate, or at 37°C for 10 min with or without 1 mM levamisole, 1 mM (R)-(−)-epinephrine, or 0.025% Triton. Cells were assessed for cellular permeability by incorporation of ethidium bromide immediately (0 h) or 3 days later (3 d) as described under Materials and Methods. Measurements were performed in triplicate, and data are mean ± SD values of three separate experiments. The number of ethidium bromide stained cells is expressed as a percentage of permeabilized cells with respect to the total. The total cells per counted field were about 200.

vor of such a conclusion are that (1) the enzyme was capable of acting on substrate in the external medium; the substrate *p*NPP is known not to penetrate the cell membrane [DePierre and Karnovsky, 1974]; (2) phosphohydrolase activity is not detected by direct measurement of the reaction buffer, ruling out the possibility that activity is a result of leakage of intracellular enzymes by secretion or cellular damage; and (3) substrate hydrolysis was linear with time, demonstrating that cell breakage did not occur during the incubation. Ecto-phosphatase activity was induced by RA in a time-dependent manner and high levels of ecto-phosphatase activity correlated with the dephosphorylation state of $^{32}$P-labeled plasma membrane proteins and a 98-kDa surface phosphoprotein in RA-treated HL-60 and P19 cells, respectively.

Using washed intact P19 and HL-60 cells incubated with *p*NPP under physiological pH conditions, we report a high degree of ecto-phosphatase activity. The ecto-phosphatase activity was inhibited by levamisole in a stereospecific and reversible manner. The data are in agreement with the inhibition by levamisole at alkaline pH of purified L/B/K ALP [Van Belle, 1976] and L/B/K ALP measured in extracts of P19 and HL-60 cells [Scheibe et al., 1991; Wei et al., 1992] (R.J. Scheibe, unpublished observation). At pH 7.4, levamisole also inhibited activity of purified or membrane-bound ALP of chick cartilage, bone, and rat liver [Cyboron et al., 1982; Farley et al., 1982; Müller et al., 1991; Sarrouilhe et al., 1993]. Levamisole did not inhibit extracellular phosphohydrolase activities of other membrane enzymes [El Kouni and Cha, 1982; LeBel et al., 1980], pointing out the specificity of levamisole toward ALP. Although the transport of levamisole across the plasma membrane cannot be excluded, the fact that the substrate *p*NPP is impermeable for membranes prevented the detection of intracellular levamisole-sensitive *p*NPP hydrolase activity in this study. Owing to the stereospecific and noncom-

petitive inhibition by levamisole, we concluded that the ecto-*p*NPP hydrolase activity detected on the surface of intact P19 and HL-60 cells represented ecto-ALP activity. It has been shown previously that PI-PLC is membrane impermeable, that it removes the glycophospholipid-linked ALP from plasma membranes, and that solubilization of ALP did not affect its ability to hydrolyze *p*NPP [Low and Finean, 1977; Pizauro et al., 1995]. We conclude that the *p*NPPase activity released into the reaction buffer after the addition of PI-PLC was attributable to the soluble form of ALP, as indicated by its sensitivity to levamisole. Furthermore, the absence of *p*NPPase activity in our reaction buffer before the addition of PI-PLC demonstrates that ALP bound to P19 and HL-60 plasma membranes was not released spontaneously as observed in osteosarcoma cells [Fukayama and Tashjian, 1990; Nakamura et al., 1988] or that intracellular ALP leaked out into the reaction buffer.

Ecto-ALP activity increased with pH and was optimal over a pH range of 7.4–8.5, using *p*NPP at micromolar concentrations. Several studies with purified ALP isozymes presented evidence that the pH optimum of the isolated enzyme depends on the kind of substrate and on substrate concentrations [Van Belle, 1976]. For example, using natural phosphomonoesters and phosphohistones as the substrates, a pH optimum of 7–9 was determined with isozymes of purified or plasma membrane-bound human liver [Chan and Stinson, 1986], human placental [Huang et al., 1976], rat liver [Ohkubo et al., 1974], bovine liver, and calf intestine ALP [Swarup et al., 1981]. In this study, we investigated the effect of substrate concentrations on the pH optimum of ecto-ALP activity and demonstrated that the rate of extracellular hydrolysis was optimal at pH 10.5, using 2 mM of *p*NPP, and decreased with decreasing concentrations of the substrate. Ecto-ALP activity on P19 and HL-60 cells was optimal within the physiological pH range at micromolar concentrations (10–250 μM) of *p*NPP. With 250 μM *p*NPP, the optimal pH did not peak; rather, it plateaued from about pH 7.8. A similar plateau of the pH optimum has been reported for phosphotyrosine hydrolytic activity of ALP on intact bone cells [Puzas and Brand, 1985], for *p*NPP hydrolase activity of ALP on intact fetal calvaria cells [Anagnostou et al., 1996], and for phosphohydrolase activity of purified rat liver ALP [Sarrouilhe et al., 1993], using micromolar substrate concentrations. The data support observations by Fedde et al. [1988] on the extracellular degradation of micromolar concentrations of several natural substrates by ALP at physiological pH, determined directly on intact human osteosarcoma cells. Similarly, Sarrouilhe et al. [1993] reported that maximal catalytic activity of isolated rat liver ALP at physiological pH was measured with *p*NPP concentrations of 10–500 μM. Thus, at micromolar substrate concentrations, ALP should no longer regarded as "alkaline."

RA is known to increase specific activity of ALP in various cells during differentiation, including the P19 and HL-60 lines, as a result of induced transcriptional activation of the *ALP* gene [Scheibe et al., 1991; Wei et al., 1992]. We have previously determined the induction of ALP activity by RA in cellular extracts at alkaline pH using 1 mM *p*NPP as substrate [Scheibe et al., 1991]. In addition, we have demonstrated that after RA-treatment for 48 h, RA increased plasma membrane-bound ALP activity, which we determined extracellularly by cytochemistry at alkaline pH (W.H. Mueller et al., unpublished results). So far, RA has been shown to modulate the phosphorylation/dephosphorylation state of cytosolic proteins or membrane proteins at its cytoplasmic domain in HL-60 [Durham et al., 1985], P19 [Oulad-Abdelghani et al., 1996] and other teratocarcinoma cells [Banerjee et al., 1986]. Also, changes in cellular protein phosphatase and kinase activities after RA-treatment have been reported in these cell lines [Durham et al., 1985; Mizuno et al., 1997]. The effect of RA on ecto-enzyme activities was only studied in differentiating neuroblastoma SH-SY5Y cells, where RA induced the ecto-5′-nucleotidase activity [Kohring and Zimmermann, 1998] and on human lung fibroblasts, where RA decreased ecto-protein kinase C activity [Gmeiner, 1987]. We now present evidence that the levamisole-sensitive ecto-ALP activity on P19 and HL-60 cells was induced by RA. Ecto-enzyme activity was determined at physiological pH and $V_{max}$ increased in RA-treated cells. The maximum induction of ALP activity was observed in P19 and HL-60 cells within 24–48 h of RA treatment, and elevated enzyme activity was found to decrease as cells differentiate [Scheibe et al., 1991; Wei et al., 1992]. A 48-h co-treatment with RA and levamisole caused a delay of cellular differentiation in both

cell types, as well as a change in P19 cell attachment to poly-D-lysine-coated culture dishes (T. Keitel, unpublished results). Thus, it is possible that ecto-ALP activity is involved in the control of plasma membrane functions early in the differentiation pathway.

Ecto-protein kinase activities have been observed on various cell types, including neurons and HL-60 cells [for a review, see El-Moatassim et al., 1992; Paas and Fishelson, 1995], and extracellular protein phosphorylation events are involved in regulating the function of receptors [Asch et al., 1993; Ehrlich et al., 1990], cell-adhesion proteins [Ehrlich et al., 1986, 1990], and growth factors [Nagashima et al., 1991; Pawlowska et al., 1993] and play a role in neuritogenesis and neuronal adhesion [Hogan et al., 1995], myoblast fusion [Chen and Lo, 1991], and fibroblast cell growth [Friedberg et al., 1995]. The reversible nature of phosphorylation/dephosphorylation processes requires both protein kinases and phosphatases. For example, an extracellular phosphorylation/dephosphorylation system on blood platelets regulates cellular functions [Naik et al., 1991; Babinska, 1996], and ALP was capable of modulating platelet activation by extracellular dephosphorylation in vivo [Hatmi et al., 1991]. The levamisole-sensitive ecto-ALP activity reported in this study dephosphorylated cell surface phosphoproteins on P19 and HL-60 cells, which were previously autophosphorylated by ecto-kinase activity. Dephosphorylation occurred in a time-dependent manner and was induced by RA. A phosphoprotein in plasma membranes of P19 cells with an apparent molecular weight of 98 kDa was sensitive to the ecto-ALP. The identity and role of the 98-kDa phosphoprotein remain unknown and is subject of further investigation. The findings of increased dephosphorylation of P19 and HL-60 cell surface proteins and exogenous substrate after RA-treatment suggests that an extracellular phosphorylation/dephosphorylation system is involved in some specialized functions during differentiation into neuronal and granulocytic cells.

## ACKNOWLEDGMENTS

We thank Drs. B. Katthab, I. Moeller-Runge, A. Rühlmann, and U. Wirth for helpful discussions and A. Jakobs for technical assistance.

## REFERENCES

Anagnostou F, Plas C, Forest N. 1996. Ecto-alkaline phosphatase considered as levamisole-sensitive phosphatase at physiological pH range during mineralization in cultured fetal calvaria cells. J Cell Biochem 60:484–494.

Asch AS, Liu I, Briccetti FM, Barnwell JW, Kwakye-Berko F, Dokun A, Goldberger J, Pernambuco M. 1993. Analysis of CD36 binding domains: ligand specificity controlled by dephosphorylation of an ectodomain. Science 262:1436–1440.

Babinska A, Ehrlich YH, Kornecki E. 1996. Activation of human platelets by protein kinase C antibody: role for surface phosphorylation in homeostasis. Am J Physiol 271:2134–2144.

Banerjee D, Pentney RJW, Chackalaparampil I, Mukherjee BB. 1986. Ability of oncogenically transformed cells to grow without anchorage correlates with phosphorylation of a group of cell surface membrane proteins. Exp Cell Res 166:442–454.

Breitman T, Collins SJ, Keene B. 1981. Terminal differentiation of human promyelocytic leukemic cells in primary culture in response to retinoic acid. Blood 57:1000–1004.

Breitman TR, Selonick SE, Collins SJ. 1980. Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoic acid. Proc Natl Acad Sci USA 77:2936–2940.

Buell G, Michel AD, Lewis C, Collo G, Humphrey PP, Surprenant A. 1996. P2X1 receptor activation in HL-60 cells. Blood 87:2659–2664.

Caswell AM, Whyte MP, Russel RGG. 1991. Hypophosphatasia and the extracellular metabolism of inorganic pyrophosphate: clinical and laboratory aspects. Crit Rev Clin Lab Sci 28:175–232.

Chambon P. 1996. A decade of molecular biology of retinoic acid receptors. FASEB J 10:940–954.

Chambon P, Olson JA, Ross AC. 1996. The retinoid revolution. Bethesda, MD: FASEB.

Chan JRA, Stinson RA. 1986. Dephosphorylation of phosphoproteins of human liver plasma membranes by endogenous and purified liver alkaline phosphatases. J Biol Chem 261:7635–7639.

Chen XY, Lo TCY. 1991. Involvement of a cell surface protein and an ecto-protein kinase in myogenesis. Biochem J 279:475–482.

Cleland WW. 1979. Methods Enzymol 63:103–138.

Cope F, Wille J. 1989. Retinoid receptor antisense DNAs inhibit alkaline phosphatase induction and clonogenicity in malignant keratinocytes. Proc Natl Acad Sci USA 89:5590–5594.

Cyboron GW, Vejins MS, Wuthier RE. 1982. Activity of epiphyseal cartilage membrane alkaline phosphatase and the effects of its inhibitors at physiological pH. J Biol Chem 257:4141–4145.

DePierre JW, Karnovsky ML. 1974. Ecto-enzymes of the guinea pig polymorphonuclear leucocyte. I. Evidence for an ecto-adenosine monophosphatase-adenosine triphosphatase, and -*p*-nitrophenyl phosphatase. J Biol Chem 249:7111–7120.

Durham JP, Emler CA, Butcher FR, Fontana JA. 1985. Calcium-activated, phospholipid-dependent protein kinase activity and protein phosphorylation in HL-60 cells induced to differentiate by retinoic acid. FEBS Lett 185: 157–161.

Eagle H. 1977. Buffer combinations for mammalian cell cultures. Science 174:500–503.

Ehrlich YH, Davis TB, Bock E, Kornecki E, Lenox RH. 1986. Ecto protein kinase activity on the external surface of intact neural cells. Nature 320:67–69.

Ehrlich YH, Hogan MV, Pawlowska Z, Naik U, Kornecki E. 1990. Ectoprotein kinase in the regulation of cellular responsiveness to extracellular ATP. Ann NY Acad Sci 603:401–416.

El Kouni MH, Cha S. 1982. Isolation and partial characterization of a 5′-nucleotidase specific for orotidine-5′-monophosphate. Proc Natl Acad Sci USA 79:1037–1041.

El-Moatassim C, Dornand J, Mani J-C. 1992. Extracellular ATP and cell signalling. Biochim Biophys Acta 1134: 31–45.

Farley JR, Puzas JE, Baylink DJ. 1982. Effect of skeletal alkaline phosphatase inhibitors on bone cell proliferation in vitro. Miner Electrolyte Metab 7:316–323.

Fedde KN, Lane CC, Whyte MP. 1988. Alkaline phosphatase is an ecto-enzyme that acts on micromolar concentrations of natural substrates at physiological pH in human osteosarcoma (SAOS-2) cells. Arch Biochem Biophys 264: 400–409.

Friedberg I, Belzer I, Oged-Plesz O, Kübler D. 1995. Activation of cell growth inhibitor by ectoprotein kinase-mediated phosphorylation in transformed fibroblasts. J Biol Chem 270:20560–20567.

Fukayama S, Tashjian AHJ. 1990. Stimulation by parathyroid hormone of $^{45}Ca^{2+}$ uptake in osteoblast-like cells: possible involvement of alkaline phosphatase. Endocrinology 126:1941–1949.

Gmeiner BM. 1987. Phorbol ester, retinoic acid and diacylglycerol decreased ecto- but increased total basal-protein kinase activity of human fibroblasts. Biochem Biophys Res Commun 143:600–608.

Gordon JL. 1986. Extracellular ATP: effects, sources and fate. Biochem J 233:309–319.

Harris H. 1990. The human alkaline phosphatases: what we know and what we don't know. Clin Chim Acta 186: 133–150.

Hatmi M, Haye B, Gavaret JM, Vargaftig BB, Jacquemin C. 1991. Alkaline phosphatase prevents platelet stimulation by thromboxane-mimetics. Br J Pharmacol 104:554–558.

Hogan MV, Pawlowski Z, Yang H-A, Kornecki E, Ehrlich YH. 1995. Surface phosphorylation by ecto-protein kinase C in brain neurons: a target for Alzheimer's β-amyloid peptides. J Neurochem 65:2022–2031.

Huang K-P, Robinson JC, Chou JY. 1976. Phosphoprotein-phosphatase activity associated with human placental alkaline phosphatase. Biochem Biophys Res Commun 70:186–192.

Hunter T. 1995. Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. Cell 80:225–236.

Jones-Villeneuve EMV, Rudnicki MA, Harris JF, McBurney MW. 1983. Retinoic acid-induced neural differentiation of embryonal carcinoma cells. Mol Cell Biol 3:2271–2279.

Kohring K, Zimmermann H. 1998. Upregulation of ecto-5′-nucleotidase in human neuroblastoma SH-SY5Y cells on differentiation by retinoic acid or phorbolester. Neurosci Lett 258:127–130.

Laemmli UK. 1970. Cleavage of structural protein during assembly of the head of bacteriophage T4. Nature 227: 680–685.

LeBel D, Poirier GG, Phaneuf S, St-Jean P, Laliberte JF, Beaudin AR. 1980. Characterization and purification of a calcium-sensitive ATP diphosphohydrolase from pig pancreas. J Biol Chem 255:1227–1233.

Low MG, Finean JB. 1977. Release of alkaline phosphatase from membranes by a phosphatidyl inositol-specific phospholipase C. Biochem J 167:281–284.

Low MG, Saltiel AR. 1988. Structural and functional roles of glycosylphosphatidylinositol in membranes. Science 239:268–275.

McBurney MW, Rogers BJ. 1982. Isolation of male embryonal carcinoma cells and their chromosome replication patterns. Dev Biol 89:503–508.

McComb RB, Bowers GN, Posen J, Posen S. 1979. Alkaline phosphatase. New York: Plenum.

Micanovic R, Bailey CA, Brink L, Gerber L, Pan Y-CE, Hulmes JD, Udenfriend S. 1988. Aspartic acid-484 of nascent placental alkaline phosphatase condenses with a phosphatidylinositol glycan to become the carboxyl terminus of the mature enzyme. Proc Natl Acad Sci USA 85:1398–1402.

Millán JL, Fishman WH. 1995. Biology of human alkaline phosphatases with special references to cancer. Crit Rev Clin Lab Sci 32:1–39.

Mizuno K, Katagiri T, Maruyama E, Hasegawa K, Ogimoto M, Yakura H. 1997. SHP-1 is involved in neuronal differentiation of P19 embryonic carcinoma cells. FEBS Lett 417:6–12.

Morris DC, Masuhara K, Takaoka K, Ono K, Anderson HC. 1992. Immunolocalization of alkaline phosphatase in osteoblasts and matrix vesicles of human fetal bone. Bone Miner 19:287–298.

Morriss-Kay GM, Sokolova N. 1996. Embryonic development and pattern formation. FASEB J 10:961–968.

Moss DW, Campbell DM, Anagnostou-Kakaras E, King EJ. 1961. Characterization of tissue alkaline phosphatases and their partial purification by starch-gel electrophoresis. Biochem J 81:441–447.

Müller K, Schellenberger V, Borneleit P, Treide A. 1991. The alkaline phosphatase from bone: transphosphorylating activity and kinetic mechanism. Biochim Biophys Acta 1076:308–313.

Nagashima K, Nakanishi S, Matsuda Y. 1991. Inhibition of nerve growth factor-induced neurite outgrowth of PC12 cells by a protein kinase inhibitor which does not penetrate the cell membrane. FEBS Lett 293:119–123.

Naik UP, Kornecki E, Ehrlich YH. 1991. Phosphorylation and dephosphorylation of human platelet surface proteins by an ecto-protein kinase/phosphatase system. Biochim Biophys Acta 1092:256–264.

Nakamura T, Nakamura K, Stinson RA. 1988. Release of alkaline phosphatase from human osteosarcoma cells by phosphatidylinositol phospholipase C: effect of tunicamycin. Arch Biochem Biophys 265:190–196.

Ng K, Gummer P, Michelangeli V, Baterman J, Mascara T, Cole W, Martin T. 1988. Regulation of alkaline phosphatase expression in a neonatal rat clonal calvarial cell strain by retinoic acid. J Bone Miner Res 3:53–61.

Ng K, Hudson P, Power B, Manji S, Gummer P, Martin T. 1989. Retinoic acid and tumor necrosis factor-α act in concert to control the level of alkaline phosphatase mRNA. J Mol Endocrinol 3:57–64.

Ohkubo A, Langerman N, Kaplan MM. 1974. Rat liver alkaline phosphatase. J Biol Chem 249:7174–7180.

Oulad-Abdelghani M, Bouillet P, Decimo D, Gansmuller A, Heyberger S, Dolle P, Bronner S, Lutz Y, Chambon P. 1996. Characterization of a premeiotic germ cell-specific cytoplasmic protein encoded by Str8, a novel retinoic acid-responsive gene. J Cell Biol 135:469–477.

Oyama VJ, Eagle H. 1978. Measurement of cell growth in tissue cultures with a phenol reagent. Proc Soc Exp Biol Med 91:35–44.

Paas Y, Fishelson Z. 1995. Shedding of tyrosine and serine/threonine ecto-protein kinases from human leukemic cells. Arch Biochem Biophys 16:780–788.

Pawlowska Z, Hogan MV, Kornecki E, Ehrlich YH. 1993. Ecto-protein kinase and surface protein phosphorylation in PC12 cells: interaction with nerve growth factor. J Neurochem 60:678–686.

Pizauro JM, Ciancaglini P, Leone FA. 1995. Characterization of the phosphatidylinositol-specific phospholipase C-released form of rat osseous plate alkaline phosphatase and its possible significance on endochondral ossification. Mol Cell Biochem 152:121–129.

Puzas JE, Brand JS. 1985. Bone cell phosphotyrosine phosphatase: characterization and regulation by calcitropic hormones. Endocrinology 116:2463–2468.

Reese D, Fiorentino G, Claflin A, Malinin T, Politano V. 1981. Rapid induction of alkaline phosphatase activity by retinoic acid. Biochem Biophys Res Commun 102:315–321.

Sarrouilhe D, LaLeggerie P, Baudry M. 1993. Alkaline phosphatase activity at physiological pH: kinetic properties and biological significance. Cell Mol Biol 39:13–19.

Sato N, Asano S, Urabe A, Ohsawa N, Takaku F. 1985. Induction of alkaline phosphatase in neutrophilic granulocytes, a marker of cell maturity, from bone marrow of normal individuals by retinoic acid. Biochem Biophys Res Commun 131:1181–1186.

Scheibe RJ, Moeller-Runge I, Mueller WH. 1991. Retinoic acid induces the expression of alkaline phosphatase in P19 teratocarcinoma cells. J Biol Chem 266:21300–21305.

Seargeant LE, Stinson RA. 1979a. Inhibition of human alkaline phosphatases by vanadate. Biochem J 181:247–250.

Seargeant LE, Stinson RA. 1979b. Phosphoester specificity of purified human liver alkaline phosphatase. Can J Biochem 57:2000–2007.

Soto AM, Sonnenschein C. 1985. The role of estrogen on the proliferation of human breast tumor cells. J Steroid Biochem 23:87–94.

Sundler R, Alberts AW, Vagelos PR. 1978. Enzymatic properties of phosphatidylinositol inositolphosphohydrolase from *Bacillus cereus*. Substrate dilution in detergent-phospholipid micelles and bilayer vesicles. J Biol Chem 253:4175–4179.

Swarup G, Cohen S, Garbers DL. 1981. Selective dephosphorylation of proteins containing phosphotyrosine by alkaline phosphatase. J Biol Chem 256:8197– 8201.

Van Belle H. 1976. Alkaline phosphatase. I. Kinetics and inhibition by levamisole of purified isoenzymes from humans. Clin Chem 22:972–976.

Van Veldhoven PP, Mannaerts GP. 1987. Inorganic and organic phosphate measurements in the nanomolar range. Anal Biochem 161:45–48.

Wei LL-L, Hui EK-W, Wei JS, Yung BY-M. 1992. Alkaline phosphatase activity during differentiation of the human promyelocytic leukemia cell line. Biochem Biophys Res Commun 182:487–494.

Whyte MP. 1989. Alkaline phosphatase: physiological role explored in hypophosphatasia. Bone Miner Res 6:175–218.