

# Growth factor-like effects of placental alkaline phosphatase in human fetus and mouse embryo fibroblasts

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**Abstract** Human placental alkaline phosphatase (PALP) is synthesized in the placenta during pregnancy and is also expressed in many cancer patients; however, its physiological role is unknown. Here we show that in human fetus fibroblasts as well as normal and H-ras-transformed mouse embryo fibroblasts PALP stimulates DNA synthesis and cell proliferation in synergism with insulin, zinc and calcium. The mitogenic effects of PALP are associated with the activation of c-Raf-1, p42/p44 mitogen-activated protein kinases, p70 S6 kinase, Akt/PKB kinase and phosphatidylinositol 3'-kinase. The results suggest that in vivo PALP may promote fetus development as well as the growth of cancer cells which express oncogenic Ras.

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**Key words:** Placental alkaline phosphatase; Insulin; Fibroblast; DNA synthesis; Mitogen-activated protein kinase

## 1. Introduction

Placental alkaline phosphatase (PALP) is a member of the alkaline phosphatase (ALP) group of enzymes which hydrolyze phosphate containing compounds at alkaline pH [1], although at neutral pH they also hydrolyze phosphatidic acid and some other substrates [2,3]. The mature PALP is a dimer of two identical glycosylated subunits [4]. The ALP family also includes the tissue non-specific (liver/bone/kidney) ALP (TNSALP), intestinal ALP (IALP) and a PALP-like enzyme, products of separate genes [5–8].

The primary source of human PALP is placenta, which synthesizes this enzyme during the second and third trimester of pregnancy. By term, PALP becomes a major ALP in the circulation [9]. In pregnant women, decreased serum level of PALP may be associated with intrauterine growth retardation [10,11], premature rupture of membranes [12], premature labor [12], or Down's syndrome pregnancies [13], although these correlations have not always been found [14]. All these observations suggest that PALP may play some role in the development of the fetus.

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**Abbreviations:** ALP, alkaline phosphatase; PALP, placental alkaline phosphatase; TNSALP, tissue non-specific alkaline phosphatase; IALP, intestinal alkaline phosphatase; uPALP, untreated PALP; hPALP, PALP pretreated at 65°C for 30 min; Ca<sup>2+</sup>, calcium; MAP, mitogen-activated protein; p70 S6K, p70 S6 kinase; PI3K, phosphatidylinositol 3'-kinase; DMEM, Dulbecco's modified Eagle's medium; MTT, (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Interestingly, PALP is also expressed in certain cancer patients [15–21], but its possible contribution to tumorigenesis has not been established. Since the growth of fetus and tumors show many common features, it is possible that increased expression of PALP alters the growth of both fetal and cancer tissues. To examine this possibility, we initiated a study to determine the effects of PALP on the growth of untransformed and H-ras-transformed mouse NIH 3T3 fibroblasts as well as human fetus fibroblasts. We have found that in these cell lines, highly purified PALP can stimulate DNA synthesis and cell proliferation via activation of various known growth regulatory enzymes.

## 2. Materials and methods

### 2.1. Materials

Human PALP, TNSALP from bovine kidney, IALP from bovine intestinal mucosa (unit of ALP activity always defined as given in Sigma catalog) and (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were bought from Sigma. Insulin was purchased from Boehringer Mannheim. [Methyl-<sup>3</sup>H]thymidine (500 mCi/mmol) and [ $\gamma$ -<sup>32</sup>P]ATP (60 Ci/mmol) were from Dupont NEN. The p42/p44 mitogen-activated protein (MAP) kinase kit was bought from New England Biolabs; the p70 S6 kinase (p70 S6K), phosphatidylinositol 3'-kinase (PI3K), Akt/PKB and c-Raf-1 assay kits were from Upstate Biotechnology. p-TYR (PY20)-agarose conjugate was from Santa Cruz Biotechnology. Tissue culture reagents, including Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum were bought from Gibco-BRL.

### 2.2. Cell culture

The NIH 3T3 clone-7 and H-ras-transformed NIH 3T3 fibroblast lines were provided by Dr. Douglas R. Louis (National Cancer Institute, National Institutes of Health, Bethesda, MD, USA) and maintained in DMEM as described previously [22]. The HTB-157 fetus (second trimester) lung fibroblast-like cells, bought from the American Type Culture Collection, were cultured in 10% fetal calf serum-containing DMEM as indicated in [22].

### 2.3. Assay of ALP activity

ALP activity was assayed spectrophotometrically by monitoring the hydrolysis of 4-nitrophenylphosphate (as an increase in absorbance at 410 nm) at room temperature (22°C) under conditions as described in [23]. The extinction coefficient of 4-nitrophenol was taken as  $1.62 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ . One unit enzyme activity is defined as 1  $\mu\text{mol}$  substrate hydrolyzed/min at 22°C at pH 9.8.

### 2.4. Purification of human PALP

A partially purified human PALP preparation from Sigma was first further purified by successive concanavalin A-Sepharose and Q-Sepharose chromatography as described by Chang et al. [24]. Finally, the Q-Sepharose PALP fraction (which still contained two major proteins) was purified to homogeneity by *t*-butyl HIC chromatography [24]. The 5 ml bed volume *t*-butyl HIC cartridge was connected to a Pharmacia FPLC system. The PALP activity was assayed as above. Purity of the enzyme was confirmed by SDS-PAGE using Coomassie blue stain.

Table 1  
Stimulatory effects of PALP and insulin on signal transducing enzymes

Addition	Enzyme activity ( $^{32}\text{P}$ -labeling (dpm)/mg protein/min)			
	p70 S6K	PI3K	Akt kinase	Raf-1 kinase
None	3410; 2850	14 820; 10 760	1 830; 1 810	850; 520
hPALP, 40 nM	11 290; 10 710	40 000; 31 620	2 110; 2 070	2 970; 2 080
hPALP, 100 nM	15 200; 14 980	45 820; 36 800	3 580; 3 330	3 560; 3 250
hPALP, 200 nM	22 050; 20 510	52 230; 52 130	8 240; 7 680	4 850; 4 740
uPALP, 200 nM	20 100; 10 010	46 330; 46 270	5 180; 4 690	3 650; 3 440
Insulin	26 360; 23 790	300 000; 256 100	126 890; 118 060	1 130; 950
hPALP, 200 nM+insulin	28 410; 26 990	288 660; 232 050	121 350; 116 670	9 850; 9 055

Serum-starved (24 h) NIH 3T3 fibroblasts were first treated with various concentrations of hPALP or uPALP, as indicated, for 2 min and then with 500 nM insulin for 5 min. This was followed by the enzyme assays as described in Section 2. Data for each enzyme activity, expressed as  $^{32}\text{P}$ -labeling of the specific substrate by 1 mg of (preimmunocomplex) protein per 1 min, are given for each of the two experiments performed.

### 2.5. Determination of DNA synthesis

Fibroblasts were grown in 12-well tissue culture dishes to about 40–50% confluency in 10% fetal calf serum-containing DMEM, washed and then incubated in serum-free medium for 27 h. The cells were washed and then treated for 17 h in serum-free medium with PALP or other ALPs in the absence or presence of insulin and/or calcium/zinc, followed by incubation for 1 h in the presence of [ $^3\text{H}$ ]thymidine (1  $\mu\text{Ci}/\text{well}$ ). In these experiments, PALP was added to the cells 10 and 20 min prior to calcium/zinc and insulin, respectively; however, PALP was equally effective when added 20 min prior to or 10 min after insulin. The cells were washed twice with PBS, then four times with 5% trichloroacetic acid and, finally, twice with absolute ethanol. The acid-insoluble material was redissolved in 0.3 M sodium hydroxide and then  $^3\text{H}$  activity was counted.

### 2.6. MAP kinase phosphorylation assay

Serum-starved NIH 3T3 fibroblasts, grown in 35-mm diameter dishes to 70–80% confluency, were incubated for 10 min with 200 nM PALP, TNSALP (1 U/ml) or IALP (1 U/ml) in the absence or presence of 500 nM insulin plus 2.8 mM calcium. Samples for Western immunoblot analysis were prepared as described earlier [25]. A phospho-specific antibody (New England Biolabs), recognizing specific, activating, phosphorylated sites in p42/p44 MAP kinases was used to quantitate the phosphorylated/activated forms of these enzymes. The relative changes in the intensity of phosphorylation were determined by a Storm 840 phosphorImager (Molecular Dynamics).

### 2.7. In vitro kinase assays

Serum-starved (24 h) NIH 3T3 cells, grown in six-well plates, were first treated with 200 nM PALP for 2 min then with 500 nM insulin for 5 min. Enzyme assays were performed for 10 min; each enzyme reaction was linear during this time period.

To determine p70 S6K activity, immunoprecipitates, prepared from cell extracts by using anti-rabbit polyclonal p70 S6 kinase antibody, were used to phosphorylate the substrate peptide AKRRRLSSLRA [26]. p70 S6 kinase activity is expressed as the amount of  $^{32}\text{P}$  (dpm) transferred from [ $\gamma$ - $^{32}\text{P}$ ]ATP to the substrate peptide per 1 mg (pre-immunocomplex) protein during 1 min incubation at 30°C.

PI3K activity was determined as described earlier [27]. Briefly, PI3K was immunoprecipitated from cell extracts with PY20-agarose conjugate and then incubated with the substrates phosphatidylinositol and [ $\gamma$ - $^{32}\text{P}$ ]ATP, followed by the separation of the product [ $^{32}\text{P}$ ]phosphatidylinositol 3-phosphate. The product was visualized and quantitatively analyzed by the Storm phosphorImager. PI3K activity is expressed as the amount of  $^{32}\text{P}$  (dpm) incorporated into phosphatidylinositol 3-phosphate per 1 mg (pre-immunocomplex) protein during 1 min incubation at 30°C.

The activity of Akt/PKB was determined in immunoprecipitates by using [ $\gamma$ - $^{32}\text{P}$ ]ATP and the RPRAATF peptide as substrates, as described previously [26]. The enzyme activity is expressed as the amount of  $^{32}\text{P}$  (dpm) incorporated into the substrate peptide per 1 mg (pre-immunocomplex) protein during 1 min incubation at 30°C.

c-Raf-1 kinase was immunoprecipitated from 0.5 ml cell lysate (250  $\mu\text{g}$  protein) with 2  $\mu\text{g}$  of anti-human c-Raf-1 antibody (Upstate Biotechnology) plus 50  $\mu\text{l}$  of protein A/G agarose (Santa Cruz Biotechnology). c-Raf-1 activity was determined by an in vitro kinase cascade reaction according to the manufacturer's (Upstate Biotechnology) in-

structions. Briefly, the immunocomplex first catalyzes phosphorylation and activation of inactive MAP kinase kinase, followed by the phosphorylation (by MAP kinase kinase) and activation of p42/p44 MAP kinases. MAP kinase activity is then determined by the phosphorylation of myelin basic protein in the presence of [ $\gamma$ - $^{32}\text{P}$ ]ATP. c-Raf-1 activity is expressed as the amount of  $^{32}\text{P}$  (dpm) incorporated into myelin basic protein per 1 mg (pre-immunocomplex) protein during 1 min incubation at 30°C.

### 2.8. Cell proliferation assay

Changes in the number of viable cells in serum-starved (24 h) NIH 3T3 cultures (cells seeded in 96-well plates at 1000 cells per well) upon treatments with PALP and insulin were determined by the MTT method [28] as described previously in detail [29]. A Labsystem Multiskan MS microplate reader was used to read absorbance and to analyze data [29].

## 3. Results

Sigma PALP was used as the starting material to obtain a completely purified enzyme fraction which, after separation by gel electrophoresis, contained only one band (Fig. 1). Eight different preparations of PALP, consistently expressing 12–15-times higher specific activity (on average, 120 U/mg protein) than the Sigma preparation, were used during this study with no detectable differences in their effects on mitogenesis and signal transduction. PALP is known to be a highly thermostable enzyme; accordingly, heating the enzyme at 65°C for 30 min did not change enzyme activity. In most experiments we used both untreated PALP (uPALP) and preheated PALP (hPALP).

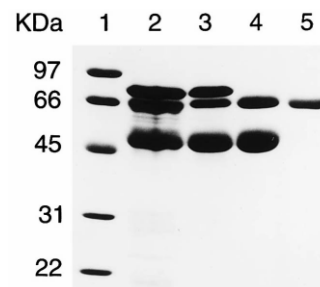


Fig. 1. Final purification of Sigma-produced PALP. Partially purified Sigma PALP preparation (lane 2; SDS-PAGE, Coomassie blue stain) was further purified by successive concanavalin A-Sepharose (lane 3), Q-Sepharose (lane 4) and *t*-butyl HIC chromatography (2–0 M ammonium sulfate gradient) (lane 5). Lane 1 shows the molecular mass standards. Each (eight) preparation of purified PALP used in this study showed a single band.

In serum-starved mouse NIH 3T3 fibroblasts, 40–200 nM concentrations of uPALP alone stimulated DNA synthesis 2.6–4.1-fold and, importantly, they also potentiated the stimulatory effect of insulin (Fig. 2A). Unexpectedly, hPALP had greater effects than uPALP on DNA synthesis (Fig. 2A). Of potential interest is that preheated PALP retained its greater mitogenic activity for at least three days at 4°C. Pretreatments at 75°C similarly increased the mitogenic activity of PALP, while pretreatments at 55 and 85°C caused 40 and 55% less activation of PALP, respectively. A PALP specific antibody (Zymed; 50 µg/ml) inhibited the stimulatory effects of 200 nM (~25 µg/ml) hPALP and uPALP on DNA synthesis about 80–85%, but it did not modify the effects of 10% serum, 500 nM insulin or 20 ng/ml fibroblast growth factor. This further confirmed that PALP, and not an invisible minor contaminant, was the source of mitogenic activity. We should add here that neither TNSALP nor IALP, used at 0.1–5 U/ml concentrations, stimulated DNA synthesis in the absence or presence of insulin (data not shown). These findings, combined with the observed increase in the mitogenic but not catalytic activity of PALP upon heating (Fig. 2A), suggest

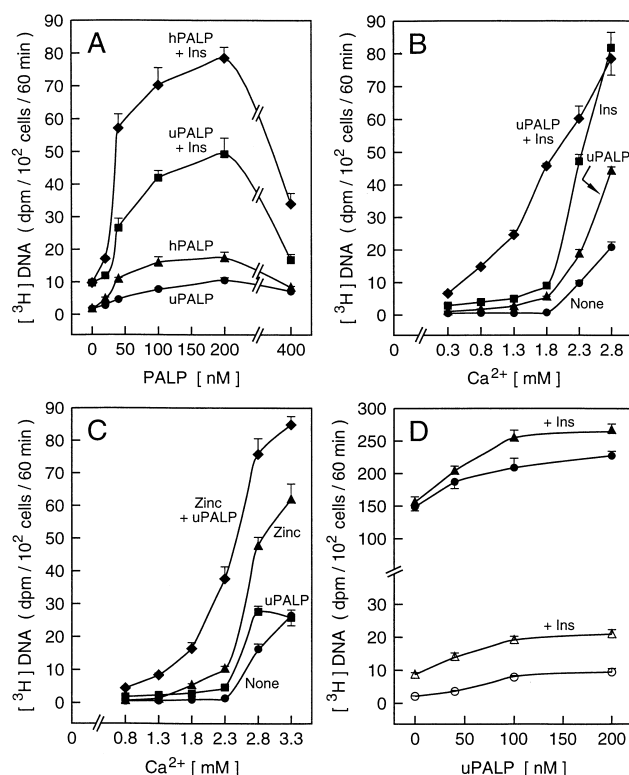


Fig. 2. PALP stimulates DNA synthesis in various cell lines. (A) Serum-starved NIH 3T3 fibroblasts were treated for 18 h with 0–400 nM uPALP or hPALP in the absence or presence of 500 nM insulin, as indicated. (B) Serum-starved NIH 3T3 fibroblasts were treated for 18 h with 200 nM uPALP ± 500 nM insulin in the presence of 0.3–2.8 mM Ca<sup>2+</sup> in the medium, as indicated. (C) Serum-starved NIH 3T3 fibroblasts were treated for 18 h with 200 nM uPALP ± 25 µM ZnCl<sub>2</sub> in the presence of 0.8–3.3 mM Ca<sup>2+</sup>, as indicated. (D) Serum-starved fetal lung fibroblasts (open symbols) and H-ras-transformed NIH 3T3 fibroblasts (closed symbols) were treated for 18 h with 200 nM uPALP ± 500 nM insulin, as indicated. In each case, error bars indicate S.D. ( $n=6$ ). Each experiment presented was repeated at least twice with essentially equivalent results.

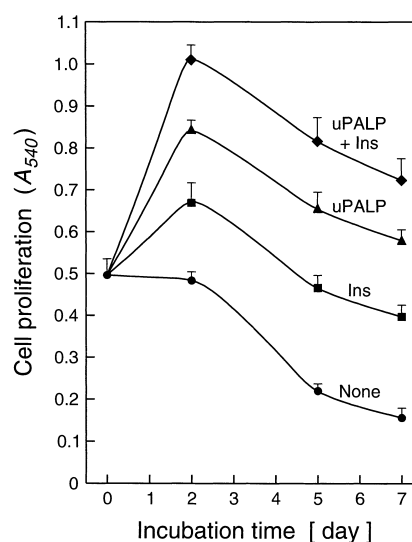


Fig. 3. Stimulatory effects of PALP and insulin on the proliferation of NIH 3T3 fibroblasts. Serum-starved (24 h) cells were incubated for up to 7 days in the absence (●) or presence of 200 nM uPALP (▲), 500 nM insulin (Ins) (■) or 200 nM uPALP plus 500 nM insulin (◆), followed by the determination of viable cells by the MTT assay as described in Section 2. Each point represents the mean ± S.E.M. of three separate experiments ( $n=16$ ).

that PALP acts on DNA synthesis by a mechanism which may not require its phosphatase activity.

The DMEM medium used for cell culture contains 1.8 mM calcium (Ca<sup>2+</sup>). As shown in Fig. 2B, maximal synergistic effects of uPALP and insulin occurred at this Ca<sup>2+</sup> concentration, but well detectable effects also were observed at 0.8–1.3 mM Ca<sup>2+</sup> concentrations. At 2.3–2.8 mM concentrations, Ca<sup>2+</sup> itself greatly enhanced the effect of insulin and PALP had only little or no additional effects (Fig. 2B). As reported earlier [30], 3.8 mM Ca<sup>2+</sup> maximally enhanced the mitogenic effect of insulin; again, under this latter condition PALP had no additional effect (data not shown).

The mitogenic activity of uPALP was also potentiated by 25 µM zinc chloride (Fig. 2C). We should note here that PALP is a zinc enzyme and during the various steps of the purification procedure the enzyme could lose some zinc. Therefore, it is possible that the potentiating effect of zinc

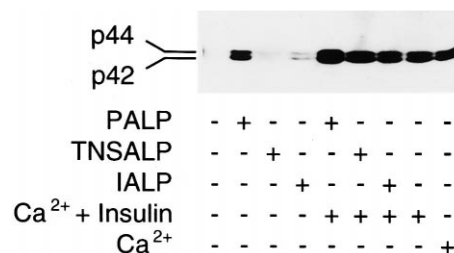


Fig. 4. Effects of ALPs, Ca<sup>2+</sup> and insulin on p42/p44 MAP kinase phosphorylation. Serum-starved NIH 3T3 cells were treated with purified (non heat-treated) PALP (200 nM) as well as Sigma TNSALP or IALP, each at 1 U/ml concentration, for 2 min, followed by treatments for 10 min with 500 nM insulin and/or 1 mM extra Ca<sup>2+</sup> (2.8 mM total Ca<sup>2+</sup>) as shown. The stimulatory effects of PALP in the absence as well as presence of insulin plus Ca<sup>2+</sup> were confirmed in three additional experiments.

merely reflects that the mitogenic effect of PALP requires enzyme bound zinc (which is absent from DMEM). Alternatively, since zinc sensitizes these fibroblasts to the mitogenic actions of  $\text{Ca}^{2+}$  [30], zinc could also act by lowering the concentration of  $\text{Ca}^{2+}$  required for the action of PALP. Further work is required to resolve this issue.

In serum-starved lung fibroblasts originally derived from a 4-month-old fetus, 200 nM uPALP stimulated DNA synthesis 5-fold and it also enhanced the insulin effect 2.3-fold (Fig. 2D). Similarly, in serum-starved Ha-Ras-transformed NIH 3T3 fibroblasts, 200 nM uPALP had 1.5–1.7-fold stimulatory effects on DNA synthesis both in the absence and presence of insulin (Fig. 2D).

Serum starvation usually elicits rapid apoptotic cell death in most fibroblast lines. However, incubation of NIH 3T3 fibroblasts in serum-free medium for 2 days did not reduce the number of viable cells as determined by the MTT colorimetric assay (Fig. 3) and verified by direct cell counting. During the first 2 days of incubation, exposure to insulin and PALP enhanced cell numbers 1.38- and 1.75-fold, respectively, while in combination they had about a two-fold effect (Fig. 3). After incubations for 5 or 7 days, the number of cells both in the untreated and treated cultures were substantially reduced with similar kinetics (Fig. 3). These data indicated that while both PALP and insulin, particularly in combination, increase cell proliferation, in this cellular system none of them can prevent serum deficiency-induced cell death.

In serum-starved NIH 3T3 and fetal fibroblasts, the effects of hPALP as well as hPALP plus insulin on DNA synthesis were inhibited 51–58% by 50  $\mu\text{M}$  PD 98059, a MAP kinase inhibitor [31], 72–83% by rapamycin (10  $\mu\text{M}$ ), a specific inhibitor of the actions of p70 S6K [32], and 20–27% by wortmannin (200 nM), an inhibitor of PI3K [33]. These data suggested that PALP may stimulate both the c-Raf-1 kinase  $\rightarrow$  MAP kinase and the p70 S6K-dependent mitogenic pathways.

PI3K and Akt/PKB often mediate the p70 S6K-activating effects of insulin and other growth factors [34–36]. In serum-starved NIH 3T3 fibroblasts, both hPALP and uPALP increased the activities of PI3K, Akt/PKB, p70 S6K and c-Raf-1 kinase (Table 1). However, insulin was more effective than hPALP in stimulating p70 S6K, and particularly PI3K and Akt/PKB activities, and none of these insulin effects were enhanced by hPALP (Table 1). Unexpectedly, insulin alone failed to activate c-Raf-1 kinase activity, yet it enhanced the stimulatory effect of hPALP (Table 1).

Activating phosphorylation of p42/p44 MAP kinases is required for mitogenesis in fibroblasts [37]. At 200-nM concentration, uPALP enhanced activating phosphorylation of p42/p44 MAP kinases 8–11-fold as determined by a phosphorImager (Fig. 4). In addition, PALP also approximately doubled the large (17–24-fold) stimulatory effects of insulin plus 2.8 mM  $\text{Ca}^{2+}$  (Fig. 4). These stimulatory effects of PALP were highly reproducible in four experiments. In contrast to PALP, neither TNSALP nor IALP affected MAP kinase phosphorylation (Fig. 4). Finally, hPALP (200 nM) was only slightly more effective than uPALP (200 nM) in stimulating MAP kinase phosphorylation (not shown).

In conclusion, we have found that in untransformed and H-ras-transformed mouse NIH 3T3 fibroblasts as well as fetal fibroblasts, PALP, particularly in combination with insulin, increases mitogenesis in a  $\text{Ca}^{2+}$ -dependent manner. Heat

treatment and zinc increase the mitogenic activity of PALP by presently unknown mechanisms. Importantly, the mitogenic activity of PALP may be independent of its phosphatase activity. The effects of PALP on DNA synthesis are associated with the activation of p42/p44 MAP kinases, p70 S6K, PI3K, Akt/PKB kinase and c-Raf-1 kinase. The significance of these enzymes in the mediation of PALP effects on DNA synthesis remains to be determined. These results suggest that PALP may be a positive regulator of fetus growth and, when expressed, it may also increase the growth of tumor cells which express oncogenic Ras.

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