*Proc. Natl. Acad. Sci. USA*
Vol. 84, pp. 4885–4889, July 1987
Biochemistry

# Expression of active, membrane-bound human placental alkaline phosphatase by transfected simian cells

**(glycolipid anchorage)**

JOEL BERGER*, ANDREW D. HOWARD*, LOUISE GERBER*, BRYAN R. CULLEN†, AND SIDNEY UDENFRIEND*‡

*Roche Institute of Molecular Biology and †Department of Molecular Genetics, Hoffmann–La Roche, Inc., Roche Research Center, Nutley, NJ 07110

*Contributed by Sidney Udenfriend, April 10, 1987*

**ABSTRACT** **Human placental alkaline phosphatase (PALPase) has been transiently expressed in simian (COS) cells by transfection with a eukaryotic expression vector containing the corresponding cDNA. The level of expression of PALPase was high, and it was produced in an enzymatically active form. The bulk of PALPase was associated with the cell membrane as shown by immunocytochemistry and subcellular fractionation studies. The PALPase produced by transfected COS cells, like PALPase in human tissue, was specifically released from the intact cells in a hydrophilic form by phosphatidylinositol-specific phospholipase C and is, therefore, apparently attached to the outer membrane by means of a phosphatidylinositol-glycan. Transfected COS cells appear to be an excellent model for elucidating the mechanism of attachment of this phosphatidylinositol-glycan to a protein moiety.**

Mammalian alkaline phosphatases [ALPases; orthophosphoric-monoester phosphohydrolases (alkaline optimum), EC 3.1.3.1] belong to a category of proteins that are anchored to the plasma membrane not by membrane-spanning peptide sequences, but by a phosphatidylinositol-glycan that is covalently linked to the mature protein (1, 2). Much of our knowledge of the chemistry of this type of linkage comes from studies on trypanosome variable surface glycoprotein (3–8), Thy-1 (9–12), and acetylcholinesterase (13–20). With the two former proteins it appears that a hydrophobic peptide is removed from the carboxyl terminus of the nascent proteins and that the glycolipid tail is then attached to the newly exposed carboxyl-terminal amino acid (4, 9, 21). This type of membrane attachment is attracting so much interest that a simplified nomenclature is needed. We suggest that the term phosphatidylinositol-glycan be abbreviated to PI-G and that proteins attached by their carboxyl terminus to a PI-G be called PI-G-tailed proteins.

In the case of ALPase it has been shown that the mature enzyme is present on plasma membranes in a form that is released by a phosphatidylinositol-specific phospholipase C (inositol phospholipase) (22–24). However, much still remains to be learned about the nature of the membrane-linking glycolipid and its site of attachment to ALPase. We felt that the latter question could best be answered by expressing ALPase in a cell line that normally does not produce the protein and then to use targeted mutagenesis of the cloned cDNA to probe the carboxyl terminus for the coupling site of the enzyme to the PI-G moiety. In this report we demonstrate that simian cells, which normally do not express human placental ALPase (PALPase), produce the enzyme in great abundance when transfected with a human PALPase cDNA expression vector. We also show that the expressed PALPase is anchored to the plasma membrane in the same manner as in human PALPase-producing cells.

## METHODS

**Cell Culture and DNA Transfection.** COS cells [originally derived from simian kidney (25)] were maintained as described (26) and were transfected using DEAE-dextran and chloroquine (27). Transfected cells were harvested 60 hr after transfection.

**Extraction of Alkaline Phosphatase.** Cells were washed twice with Dulbecco's phosphate-buffered saline and twice with homogenization buffer (10 mM Tris·HCl, pH 7.4/1 mM $MgCl_2$/20 $\mu$M $ZnSO_4$). The cells from a 100-mm dish were then scraped into 3 ml of homogenization buffer and centrifuged for 5 min at 1000 × *g* at 25°C. The supernatant was discarded, and the cells were resuspended in 500 $\mu$l of the same buffer and homogenized in a Potter–Elvehjem tissue grinder. After homogenization 214 $\mu$l of 1-butanol was added, and the mixture was Vortex mixed and incubated for 2 hr at 25°C. After centrifugation the butanol layer and interface were discarded, and the aqueous layer was utilized for biochemical and immunoblot analysis.

**Assay of Alkaline Phosphate Activity.** Enzyme activity was determined as described in the Sigma catalog with *p*-nitrophenyl phosphate as substrate. One unit of ALPase activity corresponds to 1 $\mu$mol of substrate hydrolyzed per minute at 37°C. To determine the effects of the specific amino acid inhibitors L-phenylalanine and L-homoarginine, extracts were preincubated separately with each amino acid for 10 min at 37°C prior to assay. To determine the effects of elevated temperatures, extracts were preincubated at 56°C or 65°C for the specified times (see Table 1) prior to assay.

**Immunoblot Analysis of ALPase.** NaDodSO$_4$/PAGE was performed according to Laemmli (28). Electroblotting of proteins to nitrocellulose filters and subsequent processing of the filters were carried out as described (29). Proteins were visualized by a modification of the method of Towbin *et al.* (30) using 3-amino-1-ethylcarbazole (31) (Sigma) as substrate for the horseradish peroxidase (Bio-Rad). The anti-PALPase peptide antiserum utilized here is directed against the first 9 amino acids of the amino terminus of mature PALPase (32) and is specific for the PALPase isozyme (unpublished observations).

**RNA Gel Blot Analysis of COS Cell and Placental RNA.** Total RNA was extracted by the guanidine isothiocyanate procedure followed by CsCl centrifugation (33). The RNA was electrophoresed in a 1.1% agarose gel after denaturation with glyoxal and dimethyl sulfoxide (33) and electroblotted onto a nylon membrane (Zeta-Probe; Bio-Rad). The filter was

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Abbreviations: ALPase, alkaline phosphatase; PALPase, placental alkaline phosphatase; PI-G, phosphatidylinositol-glycan.

‡To whom reprint requests should be addressed.

probed according to Thomas (34) utilizing a $^{32}$P-labeled *Kpn* I–*Taq* I PALPase cDNA fragment consisting of nucleotides 1928–2305 (35). In our hands, this fragment from the 3′-untranslated region has proved to be a highly specific probe for the PALPase transcript.

**Immunochemistry.** Immunofluorescent antibody staining of COS cells was performed as described (36). Sixty hours after transfection the cells were treated with antigen-affinity-purified rabbit anti-PALPase antibodies (DAKO, Santa Barbara, CA). This was followed by rhodamine-conjugated goat anti-rabbit antiserum (Boehringer Mannheim). Detergent was omitted from the primary antibody buffer to prevent leakage of anti-PALPase antibodies into the cells. The same anti-PALPase antibodies were used for immunoprecipitation.

**Treatment of PALPase-Transfected Cells with Phospholipases.** Purification and assay of inositol phospholipase and phosphatidylcholine-specific phospholipase C (choline phospholipase) from *Bacillus cereus* were carried out as described by Sundler *et al.* (37). The purified enzymes had activities of ≈3000 units/mg of protein, where 1 unit is defined as the amount of enzyme catalyzing the release of 0.1 μmol of inositol or choline in 2 hr at 25°C. Purified inositol phospholipase exhibited only slight (<5%) activity toward phosphatidylcholine and had no detectable protease activity.

Release of PALPase from intact cells by the phospholipases was monitored in the following manner. The cells, 60 hr after transfection, were washed three times in PBS, three times in release buffer (25 mM Tris·HCl, pH 5.0/0.25 M sucrose/10 mM glucose) and were then suspended in the same release buffer at a concentration of $5 \times 10^6$ cells per ml. Duplicate 30-μl aliquots of cell suspension were then incubated with 1 unit of inositol phospholipase, 1 unit of choline phospholipase, or buffer for various lengths of time at 37°C. Following incubation, the cells were pelleted by centrifugation at 12,000 × *g* for 2 min, and the supernatants were assayed for ALPase enzyme activity.

**Triton X-114 Partitioning of PALPase.** Suspensions of human placental particulate fraction (38, 39) and PALPase-transfected COS cells were incubated for 2 hr as above with release buffer, with either of the two phospholipases, or with 0.1 unit of bromelain (Sigma). After centrifugation, the supernatants of these incubations were subjected to phase separation in 0.5% Triton X-114 by the procedure of Bordier (40) as modified by Malik and Low (39). Samples of each phase were assayed for AP activity.

## RESULTS

**Construction of pBC12/PALPase.** pBC12BI (36) is a eukaryotic expression vector containing a bacterial origin of replication, a β-lactamase (ampicillin resistance, amp$^R$) gene, a simian virus 40 origin of replication, the Rous sarcoma virus long terminal repeat transcription control region, and the genomic rat preproinsulin gene. The latter contributes an intron and an efficient polyadenylylation signal. The vector was cleaved with *Hin*dIII and *Bam*HI and filled in with Klenow DNA polymerase I. A blunt-ended 2.3-kilobase (kb) cDNA fragment containing the entire translated region and 0.7 kb of 3′-untranslated region of human PALPase (41) was ligated into the modified pBC12BI to yield pBC12/PALPase. Throughout this report cells transfected with pBC12BI are referred to as control cells while those transfected with pBC12/PALPase are referred to as PALPase-transfected cells.

**Characterization of ALPase.** Sixty hours after transfection with either pBC12BI or pBC12/PALPase, the cells were extracted with butanol, and ALPase enzyme activity and protein concentration were assayed. Extracts of the PALPase-transfected cells had specific activities ranging from 0.50 to 0.75 unit/mg of protein compared to activities of ≤0.025 unit/mg of protein for control cells. To identify the isozymic form of the ALPase that was expressed endogenously and after transfection with pBC12/PALPase, a series of assays were performed. As shown in Table 1, ALPase that was endogenously produced by COS cells was strongly inhibited by millimolar concentrations of L-homoarginine while similar amounts of L-phenylalanine had little effect. ALPase from PALPase-transfected cells, on the other hand, was strongly inhibited by L-phenylalanine and only slightly inhibited by L-homoarginine. The endogenous ALPase activity from COS cells was completely inhibited by incubation at 56°C whereas enzyme activity from PALPase-transfected cells was only slightly affected by incubation at this temperature and could even withstand temperatures as high as 65°C. These findings show that, as expected, the small amount of ALPase that is endogenously expressed by COS cells has the characteristics of the tissue unspecific isozyme whereas the enzyme expressed by PALPase-transfected cells has properties characteristic of the human PALPase isozyme (42).

Table 1. Biochemical characterization of ALPase activity from control and PALPase-transfected COS cells

| Treatment | ALPase activity, unit/mg of protein | |
|---|---|---|
| | Control COS cells | PALPase-transfected COS cells |
| None | 0.025 | 0.50 |
| L-Phenylalanine | | |
| 5.0 mM | 0.025 | 0.15 |
| 10.0 mM | 0.025 | 0.03 |
| L-Homoarginine | | |
| 5.0 mM | 0.010 | 0.49 |
| 10.0 mM | 0 | 0.44 |
| 56°C for 15 min | 0 | 0.44 |
| 65°C for 5 min | 0 | 0.40 |

L-Homoarginine is an inhibitor of tissue unspecific ALPase; L-phenylalanine is a specific inhibitor of human PALPase. Unspecific ALPase is heat labile whereas PALPase is heat stable (42).

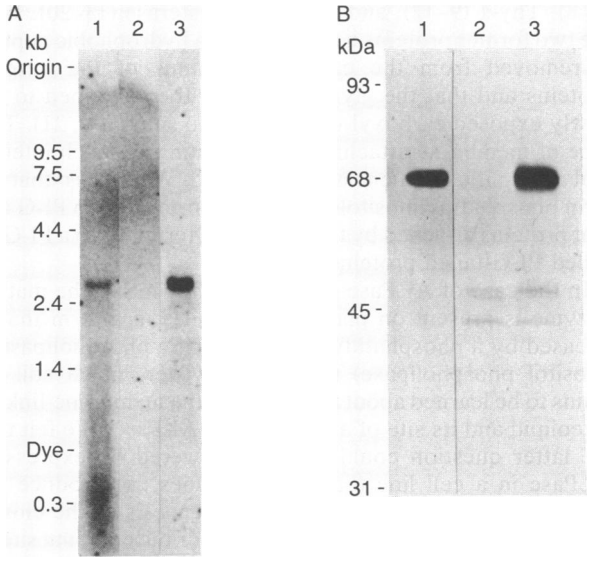


FIG. 1. (*A*) RNA gel blot analysis. Total RNA extracted from placental tissue (lane 1), from control COS cells (lane 2), and from PALPase-transfected COS cells (lane 3). The 2.8-kb band is not full-length PALPase mRNA but is a chimeric transcript containing 2.3 kb from the PALPase cDNA and 0.5 kb contributed by the rat preproinsulin gene sequences in vector pBC12/PALPase. (*B*) Immunoblot analysis. Butanol extracts of placental tissue (lane 1), control COS cells (lane 2), PALPase-transfected COS cells (lane 3). Molecular sizes in kDa are indicated.

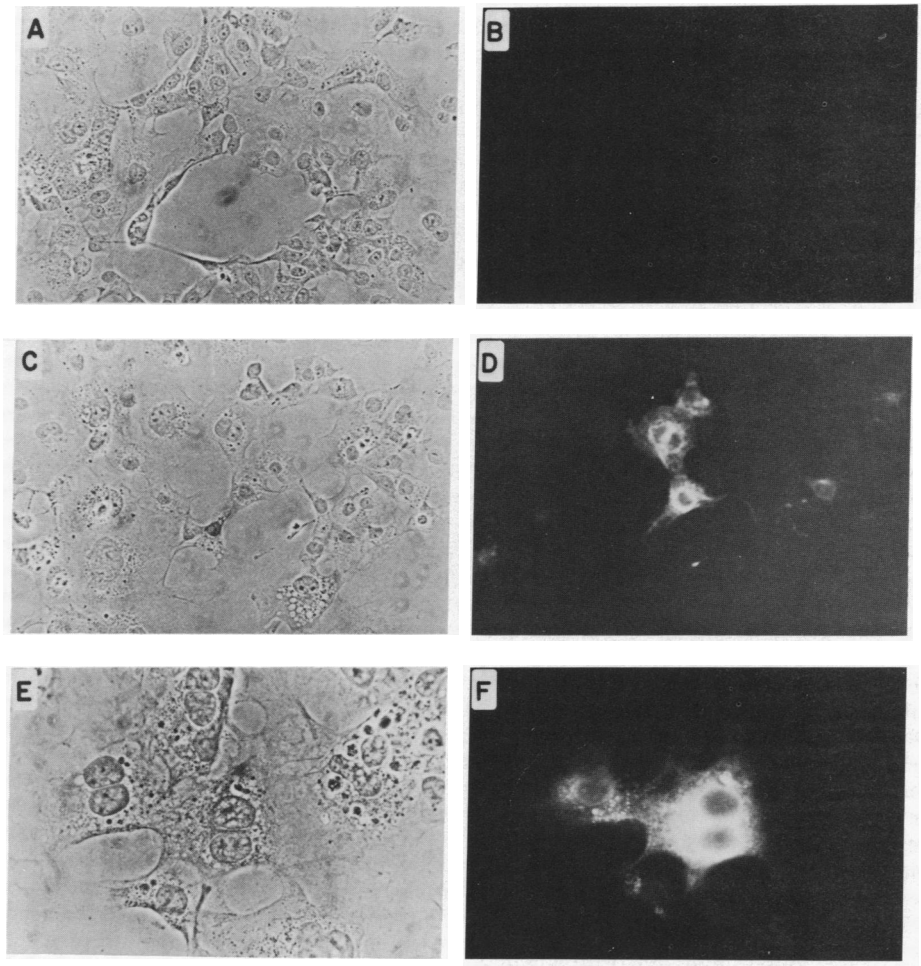


FIG. 2. Detection of human PALPase by immunofluorescent antibody staining of transfected COS cells. Paired phase and immunofluorescence photographs of control COS cells (×250, *A* and *B*); PALPase-transfected COS cells (×250, *C* and *D*) and (×630, *E* and *F*).

The nature of the ALPase expressed by PALPase-transfected COS cells was further characterized by RNA gel blot analysis. Total RNA from control or PALPase-transfected cells was probed with a $^{32}$P-labeled 0.4-kb PALPase cDNA fragment. Control cells (Fig. 1*A*, lane 2) did not contain PALPase transcripts whereas PALPase-transfected cells (lane 3) contained a single hybridizing band of 2.8 kb. Shown for comparison (lane 1) is the mRNA obtained from human placenta. The ALPase produced by PALPase-transfected cells was further characterized by immunoblot analysis. Butanol extracts of control and PALPase-transfected cells were probed with anti-PALPase antisera. Control cells did not produce detectable amounts of PALPase (Fig. 1*B*, lane 2), whereas PALPase-transfected cells yielded a strong band at ≈68 kDa (lane 3), the same molecular mass as the mature PALPase obtained from human tissue (lane 1). All these observations demonstrate that we have successfully expressed enzymatically active human PALPase in a cell line that does not normally express this protein.

**Localization of ALPase in COS Cells.** In human cells PALPase is present primarily on the outer surface of the plasma membrane (43, 44). In fact, all forms of mammalian ALPase are apparently associated with the outer cell membrane (45). To determine the location of the PALPase expressed by COS cells, the cells were examined by cell-surface immunofluorescence. Control cells did not fluoresce after immunofluorescent anti-PALP staining (Fig. 2 *A* and *B*) demonstrating the specificity of the PALPase antibodies and offering further evidence that PALPase is not expressed normally by COS cells. A subpopulation of the same cells that had been transfected with pBC12/PALPase (Fig. 2 *C* and *D*) fluoresced strongly following similar treatment. It should be noted that only a portion of the cells expressed PALPase on their surface. This was expected as only a small number of cells (≈10%) take up DNA during the transfection procedure

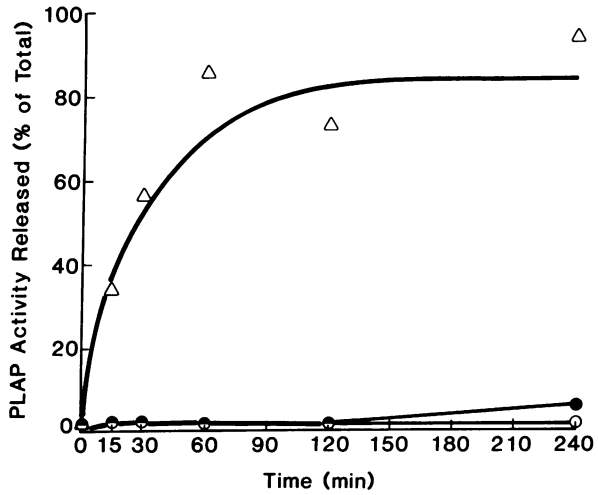


FIG. 3. Release of PALPase from pBC12/PALPase transfected COS cells by inositol phospholipase (△), choline phospholipase (●), and release buffer (○). ALPase activity was assayed in the presence of 20 mM L-homoarginine. Total PALPase activity in each sample was 2.92 milliunits.

Table 2. Triton X-114 partitioning of human PALPase released from COS cells and from placental membranes

| Treatment | PALPase-transfected COS cells | | Placental membranes | |
|---|---|---|---|---|
| | PALPase released, milliunits | Fraction in aqueous phase, % | PALPase released, milliunits | Fraction in aqueous phase, % |
| Buffer | 0.25 | 0 | 11.1 | 0 |
| Choline phospholipase | 0.25 | 0 | 10.6 | 27 |
| Inositol phospholipase | 4.54 | 100 | 23.5 | 75 |
| Bromelain | 3.78 | 100 | 57.2 | 100 |

ALPase activity was assayed in the presence of 20 mM L-homoarginine to inhibit the small amount of endogenous COS enzyme. The initial activity in each cell suspension was 4.66 milliunits. Placental membranes were actually a crude particulate fraction from frozen human placenta. The initial PALPase activity in each membrane suspension was 57.2 milliunits.

used (36). At higher magnification (Fig. 2 *E* and *F*), it can be seen that PALPase is not evenly distributed on the plasma membrane of the fixed cells but appears in definite antigenic clusters. A similar pattern of localization has been reported for endogenously expressed PALPase in cultured normal human placental and tumor-derived cells (46). The localization of PALPase to the plasma membrane was further substantiated by incubating PALPase-transfected cells with [$^{35}$S]methionine and subsequent subcellular fractionation. Approximately 90% of the labeled PALPase was found associated with the fraction that is known to be enriched with components of the plasma membrane (47).

**Means of Attachment of PALPase to the COS Cell Plasma Membrane.** It is generally believed that PALPase is attached to the plasma membrane of normal human placental cells by means of a PI-G (for reviews see refs. 1 and 2). To determine the mode of attachment of the enzyme to PALPase-transfected COS cells, the latter were incubated under three different conditions, and the release of PALPase from the cells was measured as a function of time (Fig. 3). When cells were incubated in release buffer alone or with choline phospholipase only small amounts of phosphatase activity were released, even over long periods of time. On the other hand, when the cells were incubated with inositol phospholipase large amounts of phosphatase activity were released. After 2 hr essentially all the enzyme activity of the cells was released into the buffer.

In a separate experiment, the particulate fraction from human placenta and suspensions of PALPase-transfected COS cells were each incubated with release buffer, choline phospholipase, inositol phospholipase, or bromelain. Following incubation and centrifugation, Triton X-114 was added to the supernatant. The aqueous and hydrophobic phases that appeared were separated and phosphatase activity was determined in both phases of the incubation mixtures. As shown in Table 2, PALPase from transfected COS cells and placenta, which are hydrophobic, are transformed to a hydrophilic form only by inositol phospholipase or by bromelain. The latter is an endoprotease that apparently cleaves PALPase near its carboxyl terminus (48, 49). All these findings indicate that in PALPase-transfected COS cells, as in normal placental cells, the enzyme, at a site at or near its carboxyl terminus, is apparently anchored to the outer membrane by means of the diacyl moiety of the PI-G. This conclusion was further supported by biosynthetic studies in which PALPase-transfected COS cells were incubated with [$^{14}$C]ethanolamine, [$^{3}$H]inositol, and [$^{3}$H]stearic acid, known components of the PI-G in other membrane proteins (see refs. 1 and 2). The cells were extracted, PALPase was isolated by immunoprecipitation with the specific antibodies and subjected to $NaDodSO_4$/PAGE. With each of the labeled compounds the radioactivity comigrated with authentic PALPase.

## DISCUSSION

PALPase-transfected COS cells appear to be an excellent model for investigating the details of membrane attachment of PALPase. The level of expression of the human enzyme is high and the native ALPase (the unspecific type) of the cell is present in low abundance. Furthermore, by all criteria we have examined, the mode of attachment of the human enzyme to the simian cell plasma membrane appears to be identical to that in human PALPase-producing cells. This indicates that COS cells contain the posttranslational modification mechanisms necessary to recognize nascent PALPase, process the enzyme to its PI-G-tailed form, and attach it to the plasma membrane.

In preliminary experiments, we transfected with a vector containing a mutated PALPase cDNA modified to yield a translation product shortened at its carboxyl terminus by ≈70 amino acid residues. An inactive protein was expressed that was immunoprecipitated with antibodies to human PALPase. Analysis by $NaDodSO_4$/PAGE indicated that the expression product had a lower apparent molecular weight than native PALPase. In addition, the shortened protein did not appear on the plasma membrane but remained within the cell. Such results support the conclusion that the site of attachment of the PI-G moiety to PALPase is close to the carboxyl terminus. This is in accord with findings on other PI-G-tailed membrane proteins (1, 2). Additional studies of this type on PALPase-transfected COS cells may be expected to yield data on the specific peptide chain requirements for the condensation of glycolipid to PALPase protein to occur.

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