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# Evidence Against a Role for Alkaline Phosphatase in the Dephosphorylation of Plasma Membrane Proteins: Hypophosphatasia Fibroblast Study

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**Abstract** A major impasse to understanding the physiologic role(s) of alkaline phosphatase (ALP) is uncertainty as to its natural substrates. Various in vitro studies have led other investigators to suggest that ALP functions as a plasma membrane phosphoprotein phosphatase, consistent with our demonstration of ecto-topography of ALP in a variety of cell types. Thus, we compared the phosphorylation of plasma membrane proteins from control fibroblasts to those from profoundly ALP-deficient fibroblasts of hypophosphatasia patients. Fibroblasts from 3 controls and 3 hypophosphatasia patients (ALP activity < 4% of control) were biosynthetically labeled with $^{32}P_i$ for 2 h. $^{32}P$ incorporation into total trichloroacetic acid (TCA)-precipitable material was not significantly different in control and patient cells. Plasma membranes were prepared from these cells by hypotonic shock, solubilized, and subjected to two-dimensional (2-D) gel electrophoretic separation. Video densitometric analysis of silver-stained 2-D gels failed to reveal any consistent difference in the protein profile between patient vs. control fibroblasts (i.e., unique species, altered pIs, or increased abundance). Autoradiography of individual 2-D gels demonstrated 63 plasma membrane phosphoproteins with molecular weights ranging from 15 to 152 kDa and predominantly acidic pIs. Although several of these phosphoproteins appeared to have had donor-specific labeling, none was unique or especially abundant in the hypophosphatasia group. Thus, in ALP-deficient fibroblasts, normal incorporation of $^{32}P$ into total cellular protein and into all identifiable plasma membrane phosphoproteins indicates that ALP does not modulate the phosphorylation of plasma membrane proteins. 

**Key words:** bone disease, calcium, enzymopathy, mineralization, osteomalacia, phosphate, phosphoprotein phosphatase, rickets

It is established that *intracellular* protein phosphorylation/dephosphorylation controls enzyme function, hormone- and growth factor-mediated cell responsivity, and cell division [Cohen, 1980]. However, more recently, it has become apparent that in a variety of cell types, protein kinases and phosphoproteins are located on the outer leaflet of plasma membranes as well. Thus, ecto-protein phosphorylation/dephosphorylation also appears to be of physiological importance (see Discussion).

Alkaline phosphatases [ALP; orthophosphoric-monoester phosphohydrolase (alkaline optimum) EC 3.1.3.1] are $Zn^{2+}$-containing metalloenzymes that are active as homodimers or homotetramers [Chakrabartty and Stinson, 1985; Kim and Wyckoff, 1991]. In humans, the ALPs consist of a family of four genetically distinct isoenzymes: the placental, intestinal, placental-like (or germ cell), and the so-called tissue nonspecific (TNS) or "liver/bone/kidney" ALP isoenzyme [Whyte, 1989b]. TNS-ALP exists as "secondary isoenzymes," i.e., "bone" and "liver" forms differ by post-translational modification [Whyte, 1989b]. Despite decades of research, the physiologic role of the ALPs is uncertain, partly because even at physiological pH they have "promiscuous" substrate specificity in vitro [Donella-Deana et al., 1991].

We previously demonstrated that ALP is predominantly lipid-anchored in ecto-orientation to the plasma membrane of various cell types and can function under physiologic conditions (i.e., pH) to dephosphorylate two *soluble* phosphocom-

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pounds, pyridoxal 5′-phosphate and phosphoethanolamine [Fedde et al., 1988; Fedde and Whyte, 1990]. However, investigations by others have suggested that ALP can also dephosphorylate phosphoproteins [Swarup et al., 1981; Lau et al., 1985; Chan and Stinson, 1986], although their conclusions were based primarily on in vitro investigations in which certain ALP inhibitors were used.

In this present study, we examined incorporation of $^{32}P$ into plasma membrane phosphoproteins following biosynthetic labeling of control fibroblasts and hypophosphatasia fibroblasts that were genetically deficient in TNS-ALP activity; our findings indicate that, under physiologic conditions, TNS-ALP does not regulate the phosphorylation state of plasma membrane proteins.

## MATERIALS AND METHODS

Unless otherwise indicated below, all reagents and chemicals were obtained from Sigma Chemical Company (St. Louis, MO) and were of the highest purity available.

### ALP

ALP activity was assayed using 4-methylumbelliferyl phosphate substrate as developed and described previously [Fedde and Whyte, 1990].

### Cell Culture

Fibroblasts were obtained from skin explants from three infants without known bone pathology and from three unrelated patients with the severe "perinatal" or "infantile" form of hypophosphatasia [Whyte, 1989b]. The cells had undergone 24–54 population doublings when studied. They were grown as previously described to post-confluence in 60 mm tissue culture dishes in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) in a 7.5% $CO_2$ incubator [Fedde and Whyte, 1990].

### $^{32}P$ Labeling

Confluent cultures were incubated at pH 7.4 (7.5% $CO_2$) for 1 h in carbonate-buffered saline (CBS; 44 mM carbonate, 150 mM NaCl, 1 mM $MgCl_2$) containing dialyzed (against saline) 5% FBS that had been heat-inactivated (to destroy ALP activity). Next, cultures were incubated in the same buffer, but containing 0.7 mCi/ml $^{32}P_i$ for 2 h. Then the cells were washed at 4°C 5× with Dulbecco's phosphate-buffered saline (PBS) containing 1 mM vanadate as a phosphatase inhibitor. Subsequently, plasma membranes were prepared by hypotonic shock-induced vesiculation according to Cohen et al. [1982] modified to include 1 mM vanadate in each buffer.

### Two-Dimensional (2-D) Gel Electrophoresis

The plasma membranes were solubilized in isoelectric focusing buffer and subjected to 2-D gel electrophoresis according to O'Farrell [1975]. Proteins were identified in the gels by silver stain [Merril, 1981].

### Electrotransfer

Proteins were also electrophoretically transferred to nitrocellulose sheets using a semidry electroblotter (PolyBlot, American Bionetic, Hayward, CA) [Bjerrum and Schafer-Nielsen, 1986].

### Autoradiography

$^{32}P$-labeled phosphoproteins on nitrocellulose membranes were identified by autoradiography with X-O-Mat film and one Corex-plus enhancing screen exposed at −70°C.

### Scanning Densitometry

Silver-stained gels and autoradiograms were analyzed by video densitometry (Biomed Instruments, Inc., Fullerton, CA) interfaced to an IBM AT computer. The system was equipped with the appropriate software for data acquisition and analysis (superimposition, integration, Gaussian deconvolution, molecular weight, and isoelectric point determination). Molecular weight and pI determinations were made using the QUEST [Garrels and Franza, 1989] system using recognizable proteins as standards to compare gels; tolerance (the degree to which a single protein on different gels match on the basis of molecular weight and pI) is given as percent.

## RESULTS

Fibroblasts were grown from three normal infants and three patients with severe hypophosphatasia and then labeled with $^{32}P_i$ for 2 h as described (see Materials and Methods); each culture attained a similar degree of confluence based on protein concentration per dish (Table I). The mean value for ALP specific activity in the fibroblasts from the hypophosphatasia patients was less than 4% of the control mean. Plasma membranes prepared from duplicate dishes showed similar enrichment of ALP specific activities (compared to fibroblast homogenates) from pa-

**TABLE I. Protein and ALP Activity Levels in Control and Patient Fibroblast and Plasma Membrane Preparations***

| | Culture plate | | Membrane preparation | |
|---|---|---|---|---|
| | Protein (μg/culture) | ALP (pmol/min/mg) | ALP (pmol/min/mg) | Enrichment (fold) |
| Control 1 | 350 | 20,000 | 135,000 | 6.8 |
| Control 2 | 160 | 4,000 | 31,700 | 7.9 |
| Control 3 | 780 | 12,500 | 105,000 | 8.4 |
| Mean ± S.E.M. | 430 ± 184 | 12,200 ± 4,600 | 90,600 ± 30,700 | 7.7 ± 0.47 |
| Patient 1 | 180 | 300 | 2,500 | 8.3 |
| Patient 2 | 420 | 800 | 1,800 | 2.5 |
| Patient 3 | 1,150 | 100 | 1,600 | 12.3 |
| Mean ± S.E.M. | 583 ± 292 | 390 ± 190 | 1,970 ± 270 | 7.7 ± 2.8 |

*Protein and ALP specific activity levels were determined in fibroblast homogenates and in plasma membrane preparations from 3 controls and 3 hypophosphatasia patients.

**TABLE II. $^{32}P$ Incorporation Into TCA-Precipitable Protein†**

| | fmol $^{32}P_i$/mg protein |
|---|---|
| Control 1 | 81.7 |
| Control 2 | 81.3 |
| Control 3 | 98.3 |
| Mean ± S.E. | 87 ± 5.6 |
| Patient 1 | 142 |
| Patient 2 | 70 |
| Patient 3 | 100 |
| Mean ± S.E. | 104* ± 21 |

†Fibroblast cultures from 3 controls and 3 hypophosphatasia patients were labeled with $^{32}P_i$ as described and analyzed for phosphate incorporation (into TCA-precipitable protein).
*$P$ = 0.48 (not significant).

tient and control plasma membrane preparations (mean = 7.7-fold). Accordingly, the mean ALP specific activity in patient plasma membrane preparations remained ≈5% of the control plasma membranes.

As shown in Table II, $^{32}P$ incorporation into total fibroblast trichloroacetic acid (TCA)-precipitable material was similar in control and patient cultures (87 ± 5.6 and 104 ± 21 fmol $^{32}P_i$/mg protein, respectively, $P$ = 0.48). These results demonstrated that the ALP deficiency in hypophosphatasia fibroblasts did not result in a generalized change in phosphorylation of *total* cellular phosphoproteins.

$^{32}P$-labeled plasma membranes were subjected to 2-D gel electrophoresis and were analyzed video densitometrically for proteins by silver staining and for phosphoproteins by autoradiography. Although more than 200 plasma membrane proteins were identified by silver staining, there was no consistent difference between the protein profiles of the group of control and patient preparations. Table III shows the molecular weight, isoelectric point, and relative abundance data of the proteins from the midsection of a representative 2-D gel from one patient and one control (Fig. 1 shows the scan). The molecular weights and isoelectric points varied, on the average, less than 1.5% and 0.01 pH units, respectively; the differences between controls and patients showed no trend with respect to the direction of change.

2-D autoradiograms of $^{32}P$-labeled plasma membrane preparations from each control and each patient were analyzed by video densitometry. As shown in Table IV, although no patient or control plasma membrane preparation had all 63 phosphoproteins, there was no labeled phosphoprotein present (or absent) in each patient preparation that was missing (or present) in each of the control preparations. Furthermore, there were no phosphoproteins which appeared to be consistently more abundant in the patient vs. control preparations.

## DISCUSSION

Phosphoproteins are preeminent regulators of *intracellular* processes. Among the growing list of cell functions regulated by phosphoproteins are rhodopsin-mediated vision [Yang et al., 1991], potassium channels [Carl et al., 1991] and calcium channels [Kranias et al., 1988], regulated secretion [Taffs et al., 1991; Wu and Wagner, 1991], collagenase production [Kim et al., 1990], insulin-stimulated pyruvate dehydrogenase activity [Rutter et al., 1991], cell division [Gruppuso et al., 1991a,b; Marvel et al., 1991], malignant transformation [Nagao et al., 1989],

**TABLE III. Analysis of Control and Patient Fibroblast Plasma Membrane Proteins, 2-D Gel Mid-Region***

| Plasma membrane protein no. | Patient | | | Control | | | Tolerance | | |
|---|---|---|---|---|---|---|---|---|---|
| | Abundance | pI | Mr | Mr | pI | Abundance | Mr | pI | Mr + pI |
| 40 | 0.42 | 4.45 | 32.4 | 32.7 | 4.53 | 0.55 | 0.92 | 1.77 | 2.68 |
| 44 | 2.09 | 5.81 | 30.9 | | | | | | |
| 45 | 2.15 | 5.24 | 30.9 | 31.7 | 5.26 | 2.94 | 2.52 | 0.38 | 2.90 |
| 46 | 1.52 | 4.33 | 30.4 | 30.7 | 4.19 | 0.75 | 0.98 | 3.34 | 4.32 |
| 49 | 2.52 | 4.65 | 28.5 | 28.3 | 4.69 | 1.60 | 0.71 | 0.85 | 1.56 |
| 50 | 1.95 | 4.33 | 27.6 | 28.3 | 4.27 | 1.39 | 2.47 | 1.40 | 3.88 |
| 51 | 1.96 | 5.90 | 27.2 | 27.5 | 5.88 | 0.94 | 1.09 | 0.34 | 1.43 |
| 55 | 0.54 | 5.67 | 25.5 | 26.6 | 5.54 | 0.38 | 4.14 | 2.35 | 6.48 |
| 56 | 2.12 | 4.58 | 25.1 | 25.4 | 4.61 | 1.90 | 1.18 | 0.65 | 1.83 |
| 58 | 0.12 | 5.81 | 24.3 | 24.2 | 5.63 | 2.21 | 0.41 | 3.20 | 3.61 |
| 59 | 0.61 | 5.81 | 23.1 | 22.7 | 5.68 | 0.13 | 1.76 | 2.29 | 4.05 |
| 61 | 1.71 | 4.91 | 22.7 | 23.4 | 5.03 | 1.98 | 2.99 | 2.38 | 5.38 |
| 60 | 2.34 | 4.58 | 22.7 | 23.1 | 4.61 | 3.08 | 1.73 | 0.65 | 2.38 |
| 64 | 2.38 | 5.95 | 22.4 | | | | | | |
| 65 | 1.47 | 5.24 | 21.7 | 21.7 | 5.26 | 1.85 | 0.00 | 0.38 | 0.38 |
| 68 | 4.10 | 4.61 | 20.6 | 20.7 | 4.61 | 1.84 | 0.48 | 0 | 0.48 |
| 69 | 0.30 | 5.15 | 20.6 | 20.7 | 5.26 | 0.41 | 0.48 | 2.09 | 2.57 |

*2-D gels of fibroblast plasma membrane preparations were analyzed by video densitometry. Data collected from the mid-region of one control and one patient are shown. Identity of proteins between control and patient preparations is made by isoelectric point and molecular weight matching. Tolerance (difference) is expressed as percent.

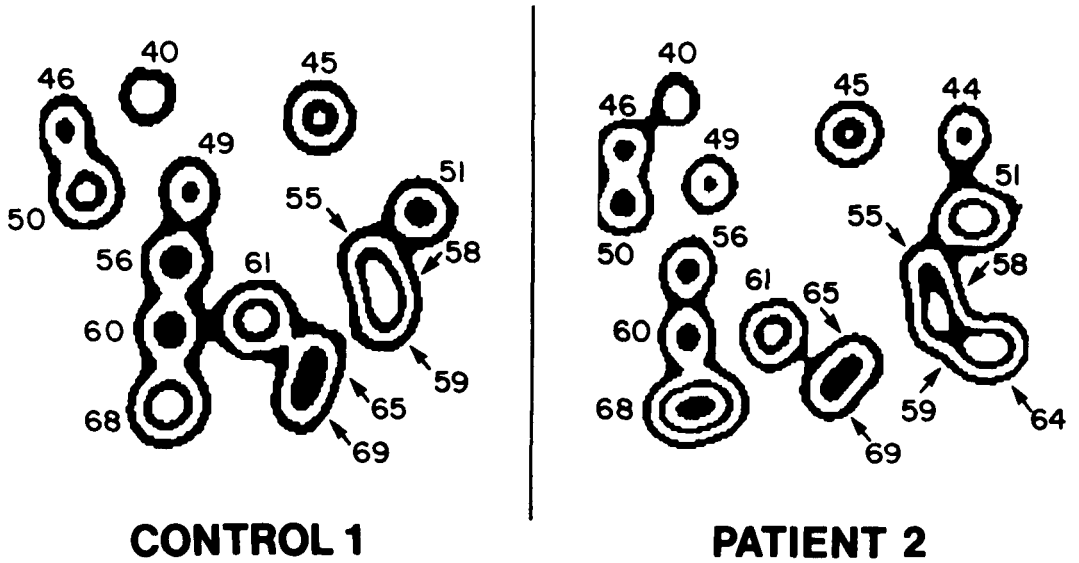


**Fig. 1.** Plasma membrane proteins (silver stained) from control and patient fibroblasts, 2-D gel mid-regions. This is a computer-generated graphic representation of the mid-region gel (one control and one patient).

macrophage motility [Wilson et al., 1991], microtubule assembly [Yamamoto et al., 1988], and angiogenesis [Montesano et al., 1988]. Phosphoproteins that act in these processes are themselves regulated by specific kinases and phosphatases which, in turn, exist in active and inactive forms.

There is also, however, growing evidence for the presence of phosphoproteins that have important extracellular functions, such as platelet activation and secretion [Naik et al., 1991], renal brush border membrane ATPase [Hilden and Madias, 1990], spermatozoa flagella motility [Barua and Majumder, 1987; Dey and Majum-

**TABLE IV. Analysis of Fibroblast Plasma Membrane Phosphoproteins From Controls and Hypophosphatasia Patients***

| Phospho-protein no. | Mr | pI | Controls 1 | Controls 2 | Controls 3 | Patients 1 | Patients 2 | Patients 3 |
|---|---|---|---|---|---|---|---|---|
| 1 | 130.4 | 4.67 | X | • | X | X | X | • |
| 2 | 109.4 | 4.59 | X | X | X | X | X | X |
| 3 | 109.4 | 5.15 | X | X | X | • | X | X |
| 4 | 107.9 | 4.03 | X | X | X | X | X | X |
| 5 | 103.6 | 6.15 | • | X | • | • | • | X |
| 6 | 98.2 | 4.23 | X | X | X | X | X | X |
| 7 | 96.8 | 3.93 | X | X | X | • | X | X |
| 8 | 91.8 | 5.23 | • | • | X | X | • | X |
| 9 | 81.3 | 5.67 | • | • | X | • | X | X |
| 10 | 78.0 | 4.37 | • | • | • | • | • | X |
| 11 | 74.9 | 7.24 | • | X | • | • | • | • |
| 12 | 73.9 | 7.86 | X | X | X | X | X | X |
| 13 | 67.3 | 8.18 | X | • | • | • | • | • |
| 14 | 67.3 | 5.27 | X | X | X | • | X | X |
| 15 | 64.6 | 6.05 | X | X | X | X | X | X |
| 16 | 62.9 | 6.84 | • | • | • | • | • | X |
| 17 | 57.2 | 5.23 | • | X | X | X | X | • |
| 18 | 57.2 | 4.90 | X | X | X | X | X | X |
| 19 | 51.3 | 4.98 | X | X | X | X | X | X |
| 20 | 50.0 | 7.01 | X | X | • | • | • | X |
| 21 | 49.3 | 8.12 | X | X | X | X | X | X |
| 22 | 45.5 | 8.45 | • | • | • | • | X | • |
| 23 | 43.7 | 7.73 | X | • | • | • | X | X |
| 24 | 41.9 | 5.67 | X | X | X | X | X | X |
| 25 | 41.9 | 6.05 | X | X | X | X | X | • |
| 26 | 39.2 | 3.97 | X | X | X | X | X | X |
| 27 | 39.2 | 6.46 | X | X | X | X | • | • |
| 28 | 37.6 | 4.67 | X | • | X | • | X | X |
| 29 | 37.6 | 6.01 | X | X | X | X | X | X |
| 30 | 34.7 | 6.36 | • | • | • | • | X | • |
| 31 | 33.8 | 5.67 | • | • | • | X | • | • |
| 32 | 33.3 | 4.37 | • | • | X | • | • | • |
| 33 | 32.9 | 6.62 | X | X | X | X | X | X |
| 34 | 30.3 | 6.15 | X | • | X | • | • | X |
| 35 | 30.3 | 4.23 | X | X | X | • | X | X |
| 36 | 29.9 | 4.74 | X | X | X | X | X | X |
| 37 | 29.9 | 4.94 | X | X | X | X | X | X |
| 38 | 29.9 | 5.77 | • | • | • | X | • | • |
| 39 | 29.9 | 4.55 | X | X | X | X | X | X |
| 40 | 27.2 | 4.13 | • | X | X | • | X | X |
| 41 | 26.8 | 4.90 | X | X | X | X | X | X |
| 42 | 26.1 | 6.25 | X | • | X | • | • | X |
| 43 | 25.1 | 5.72 | X | X | • | X | X | • |
| 44 | 25.1 | 6.84 | X | X | • | X | • | • |
| 45 | 25.1 | 4.48 | • | • | X | X | • | X |
| 46 | 24.8 | 7.79 | X | X | X | X | X | X |
| 47 | 23.8 | 5.36 | X | X | X | X | X | X |
| 48 | 22.5 | 8.12 | • | X | X | • | • | • |
| 49 | 21.9 | 7.12 | X | • | • | X | • | X |
| 50 | 21.3 | 8.59 | • | • | X | X | • | X |
| 51 | 20.2 | 4.06 | • | X | X | X | • | X |
| 52 | 19.4 | 5.81 | • | X | X | X | X | X |
| 53 | 18.6 | 7.24 | X | • | • | • | • | X |
| 54 | 18.6 | 7.67 | • | • | • | • | • | X |
| 55 | 18.6 | 9.02 | X | X | X | X | X | X |
| 56 | 18.6 | 6.84 | X | X | • | X | • | X |
| 57 | 17.7 | 6.10 | X | X | X | • | X | X |
| 58 | 17.0 | 7.67 | • | • | X | • | • | X |
| 59 | 16.1 | 8.80 | • | • | X | • | X | • |
| 60 | 16.1 | 6.68 | • | • | X | • | • | • |
| 61 | 15.9 | 5.49 | • | • | X | • | X | • |
| 62 | 15.4 | 7.36 | X | X | X | • | X | X |
| 63 | 15.2 | 7.12 | X | X | X | X | X | X |

*Plasma membranes were prepared from $^{32}P$-labeled fibroblasts and subjected to 2-D gel electrophoresis and autoradiography. Phosphoproteins are identified numerically and by molecular weight and isoelectric point. X, presence in 3 control and 3 patient plasma membranes; •, absence.

der, 1990], spermatozoa maturation [Mitra and Majumder, 1991], and smooth muscle contraction of the vas deferens [Lamport-Vrana et al., 1991]. Regulation of these ecto-phosphoproteins is also modulated by their phosphorylation state, apparently by ecto-kinases and by ecto-phosphatases.

With respect to the TNS-ALP isoenzyme, a variety of biochemical and enzymatic studies have indicated that it has a role in bone mineralization [Whyte, 1989a]. This relationship now seems certain since in hypophosphatasia, defective mineralization (rickets or osteomalacia) without exception thus far (n = 9), is caused by missense mutations in the TNS-ALP gene resulting in the production of inactive TNS-ALP [Henthorn et al., 1992; Weiss et al., 1988].

Our in vivo and in vitro observations, using the hypophosphatasia model for TNS-ALP deficiency, have indicated that TNS-ALP can function at physiologic pH to dephosphorylate pyridoxal 5′-phosphate (PLP), phosphoethanolamine (PEA), and $PP_i$ at physiologic concentrations. Although these observations indicate a physiologic role for ALP and perhaps implicate its natural substrates PLP, PEA, and $PP_i$ in bone formation, they do not define the mechanism of action.

Theories concerning the physiologic role of TNS-ALP suggest that it functions as a trans-

porter across the plasma membrane or has other intracellular actions. However, in 1988 and 1990, respectively, we demonstrated that human SaOS-2 (osteosarcoma) cells and dermal fibroblasts contain TNS-ALP that is predominantly anchored by a phosphatidylinositol glycan moiety to the outside of the plasma membrane [Fedde et al., 1988; Fedde and Whyte, 1990]. One obvious conclusion from these studies is that ALP does not act, as has been suggested, like a typical transporter or directly serve an intracellular function. Thus, it is apparent that the in vitro studies (previously cited) which demonstrated ALP activity toward such compounds as glycogen synthase, acetyl-CoA carboxylase, histone, and phosphorylase kinase must be interpreted with caution, for they appear to yield *little insight* into the physiologic function of ALP.

The in vitro studies of a number of laboratories have also demonstrated that purified ALP can remove phosphate from seryl, tyrosyl, or threonyl residues of liver plasma membrane phosphoproteins [Swarup et al., 1981; Chan and Stinson, 1986]. These results were interpreted to indicate a physiologic role for ALP in the *modulation of the phosphorylation state of* plasma membrane phosphoproteins, at least in hepatic cells. Because the topography of the bone and liver forms of TNS-ALP is the same (i.e., ecto-enzymes) [Fedde et al., 1988; Hawrylak and Stinson, 1988], we tested the hypothesis that bone ALP may function physiologically as a plasma membrane phosphoprotein ecto-phosphatase. If true, we would have expected that our ALP-deficient (hypophosphatasia) fibroblast plasma membrane preparations would contain: 1) one or more unique $^{32}$P-labeled proteins; 2) a $^{32}$P-labeled phosphoprotein(s) with an altered pI; or 3) increased labeling of phosphoproteins. None of the patient phosphoproteins that we identified fit the above criteria. Accordingly, our findings indicate that TNS-ALP does not function as a plasma membrane phosphoprotein phosphatase. Additionally, based on four lines of evidence, we believe that this conclusion can also be extended to exclude a role for TNS-ALP in dephosphorylating plasma membrane proteins in ecto-orientation, the most likely topography for substrates of an ecto-enzyme [Fedde et al., 1988; Fedde and Whyte, 1990]: 1) Although we have not further characterized the phosphoproteins in our plasma membrane preparations with respect to membrane topography, 1-D analyses following treatment of intact plasma membrane vesicles with a variety of exogenous phosphatases (commercial purified preparations of bovine intestinal ALP protein, tyrosine phosphatase 1B, and phosphatase 2B) indicated the presence of $^{32}$P-labeled ecto-phosphoproteins. This observation was consistent with studies of others showing very rapid labeling of the endogenous ATP pool (the most common substrate for phosphoprotein-kinases) by $^{32}P_i$ [King et al., 1989; Lowery et al., 1989; Tynes et al., 1988]. 2) In data not shown, after prolonged (18 h) biosynthetic labeling with $^{32}P_i$, examination of 2-D autoradiograms from plasma membranes prepared from fibroblasts also failed to reveal any control vs. patient differences (although the relative abundance of the $^{32}$P signals differed from the data shown here). 3) No changes in pIs were noted after densitometric analyses of over 200 proteins visualized by silver staining, of which a subset would be ecto-phosphoproteins. 4) Previously, we examined the role of endogenous ALP on phosphoproteins of matrix vesicles derived from a human osteosarcoma (SaOS-2) [Fedde, 1992]. In these studies, using $^{32}$P-ATP and endogenous protein kinase, we identified several ecto-phosphoproteins, none of which had a half-life affected by ALP inhibitors. We interpreted these results to indicate that ALP does not function to modulate ecto-phosphoproteins of matrix vesicles.

Support for our conclusion has recently come from the studies of Sarroulihe et al. [1992]. Using purified rat liver plasma membranes, they examined the effects of ALP inhibitors on the phosphorylation state of approximately 10 proteins identified by 1-D gel electrophoresis labeled by endogenous protein kinases. Their experiments indicated that only one plasma membrane protein (18 kDa) was regulated (directly or indirectly) by endogenous ALP. We did not observe increased labeling of an 18 kDa protein. Perhaps, their findings were due to tissue differences (liver-specific), an artifact of the plasma membrane preparations, or the labeling procedure used ($^{32}$P-ATP labeling of their preparations vs. intact cell biosynthetic labeling with $^{32}P_i$ in our study).

The only other published study that we are aware of that used the hypophosphatasia fibroblast model to address the role of ALP in phosphate metabolism was that of Eto and Tada [1988]. In their brief report, fibroblasts cultured from either one or two (not apparent) affected

siblings with lethal hypophosphatasia had approximately a onefold increase in $^{32}P$ accumulation compared to an undetermined number of control cells. It is, therefore, not clear from their study whether the observed differences were beyond the normal distribution of control fibroblasts. Their report also shows a concomitant increase in a low molecular weight $^{32}P$-labeled peak from a fast protein liquid chromatographic (FPLC) gel filtration column in patient cells. Interpretation of this result is further confounded by the fact that there were major differences in the $A_{280}$ elution patterns. Indeed, we previously found no defect in $^{32}P$ accumulation in metabolically labeled ($^{32}P_i$) fibroblasts from patients who were severely affected by hypophosphatasia [Whyte and Vrabel, 1983].

Although we cannot exclude the possibility of a physiologically important plasma membrane phosphoprotein substrate for ALP, it would appear that the phosphorylation state of the predominant plasma membrane phosphoproteins is not regulated by TNS-ALP. Thus, the search for the physiologic function of ALP, which has proceeded from regulating intracellular processes to modulating ecto-plasma membrane phosphoproteins, must now turn to a role in conditioning the extracellular matrix or soluble substrates in the extracellular milieu. With respect to soluble substrates, $PP_i$ is an inhibitor of skeletal mineralization and its extracellular accumulation in hypophosphatasia might explain the skeletal disease. However, acidic phosphoproteins secreted by the osteoblasts (including osteopontin, bone sialoprotein, and bone acidic glycoprotein-75) are believed to serve an important function in mineralization, either as a calcium-binding site or as a source of phosphate when acted upon by ALP [Gorski, 1992]. Thus, it will be important to determine the role of extracellular phosphoproteins as substrates for TNS-ALP and their function in biomineralization.

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