

High Lateral Mobility of Endogenous and Transfected Alkaline Phosphatase: A Phosphatidylinositol-anchored Membrane Protein

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Abstract. The lateral mobility of alkaline phosphatase (AP) in the plasma membrane of osteoblastic and nonosteoblastic cells was estimated by fluorescence redistribution after photobleaching in embryonic and in tumor cells, in cells that express AP naturally, and in cells transfected with an expression vector containing AP cDNA. The diffusion coefficient (D) and the mobile fraction, estimated from the percent recovery ($\%R$), were found to be cell-type dependent ranging from $(0.58 \pm 0.16) \times 10^{-9} \text{ cm}^2\text{s}^{-1}$ and 73.3 ± 10.5 in rat osteosarcoma cells ROS 17/2.8 to $(1.77 \pm 0.51) \times 10^{-9} \text{ cm}^2\text{s}^{-1}$ and 82.8 ± 2.5 in rat osteosarcoma cells UMR106. Similar values of $D \geq 10^{-9} \text{ cm}^2\text{s}^{-1}$ with $\sim 80\%$ recovery were also found in fetal rat calvaria cells, transfected skin fibroblasts, and transfected AP-negative osteosarcoma cells ROS 25/1. These values of D are many times greater than "typical" values for

membrane proteins, coming close to those of membrane lipid in fetal rat calvaria and ROS 17/2.8 cells ($D = [4-5] \times 10^{-9} \text{ cm}^2\text{s}^{-1}$ with 75–80% recovery), estimated with the hexadecanoyl aminofluorescein probe. In all cell types, phosphatidylinositol (PI)-specific phospholipase C released 60–90% of native and transfection-expressed AP, demonstrating that, as in other tissue types, AP in these cells is anchored in the membrane via a linkage to PI. These results indicate that the transfected cells used in this study possess the machinery for AP insertion into the membrane and its binding to PI. The fast AP mobility appears to be an intrinsic property of the way the protein is anchored in the membrane, a conclusion with general implications for the understanding of the slow diffusion of other membrane proteins.

ALKALINE phosphatase (AP)¹ is a membrane-bound glycoprotein, abundant in bone, liver, kidney, placenta, and intestine (McComb, 1979). AP was shown to be released as a soluble enzyme from liver (Ohyabu et al., 1978; Chakrabarty et al., 1985) and kidney (Ohyabu et al., 1978) by phosphatidylinositol (PI)-specific phospholipase C (PI-PLC). AP is seemingly one of the proteins anchored to the membrane lipid bilayer via a PI linkage, (Low et al., 1986), similar to 5' nucleotidase (Taguchi et al., 1985), acetylcholine esterase (Low and Finean, 1977), the *Trypanosoma* variant surface glycoprotein (Ferguson et al., 1985), Thy-1 (Low and Kincade, 1985), and NCAM (He et al., 1986).

The lateral mobility of membrane proteins reflects their molecular interactions with other cell components (Koppel et al., 1981; Vaz et al., 1984) and has been implicated in numerous cellular functions (Axelrod, 1983; Pollerberg et al., 1986). The lateral mobility has been measured for many membrane proteins in cell membranes, and for most, the diffusion coefficient was found to be around $2 \times 10^{-10} \text{ cm}^2\text{s}^{-1}$

or lower (Peters, 1981; Gall and Edelman, 1981); considerably slower than that found in artificial lipid bilayers (Vaz et al., 1984). Exceptionally rapid diffusion (i.e., $D > 10^{-9} \text{ cm}^2\text{s}^{-1}$) was observed in several cases (Poo and Cone, 1974; Sheetz et al., 1980; Golan and Veatch, 1980; Tank et al., 1982; Wu et al., 1982; Woda and Gilman, 1983; Myles et al., 1984; Cowan et al., 1987), and was often attributed to the absence of the more typical constraints on mobility imposed by interactions of cytoplasmic portions of the proteins with underlying cytoskeletal structures. In contrast, it was recently demonstrated that excision of major cytoplasmic portions of the H2-L^d antigens (Edidin and Zuniga, 1984), and epidermal growth factor receptors (Livneh et al., 1986) did not release constraints on lateral diffusion, leading to the conclusion that these constraints are caused by interactions within the lipid bilayer or in the extracellular regions.

The recent discovery of membrane proteins anchoring in the lipid bilayer via PI linkages offers new probes for studying the nature of the molecular interactions restricting diffusion. Because these proteins lack both cytoplasmic and bilayer spanning portions, they provide unique tests of the role that the extracellular domain may play in controlling diffusion rates. Indeed, it has been shown that Thy-1 diffuses extraordinarily rapidly in lymphocytes and fibroblasts, with

1. *Abbreviations used in this paper:* AP, alkaline phosphatase; FRAP, fluorescence redistribution after photobleaching; HDAF, hexadecanoyl aminofluorescein; PI, phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C.

$D = (2-4) \times 10^{-9} \text{ cm}^2\text{s}^{-1}$ (Woda and Gilman, 1983; Ishihara et al., 1987; the former measurement, incidentally, being performed before the nature of the PI linkage was known). If it is found that the majority of PI-linked proteins diffuse rapidly in many cell types, then it becomes increasingly unlikely that the diffusion of other membrane proteins, as a rule, is restricted by interactions within the extracellular region.

To evaluate if increased lateral mobility is found in other PI-linked membrane proteins we measured the lateral mobility of AP in a series of AP-rich bone-derived cells, and in cells that expressed high AP levels after transfection with a vector containing rat AP cDNA. The AP-PI linkage was confirmed in both cases by the release of AP from these cells with PI-PLC and the lateral mobility was found to be similar to that of Thy-1 antigen, faster than that of other proteins. To check further if AP diffusion rates could be correlated with known perturbations of cell physiology, we also examined the effects of changing cell density, and dexamethasone and $1,25(\text{OH})_2\text{D}_3$ treatments.

Materials and Methods

Culture medium (powdered Ham's F12), FBS, horse serum, and kanamycin were purchased from Gibco (Grand Island, NY). Tissue culture dishes were obtained from Costar (Cambridge, MA) and Nunc (Roskilde, Denmark). Fluorescein-conjugated monovalent antibodies were purchased from Cappel Laboratories (Malvern, PA). 5'-(hexadecanoyl)*N*-aminofluorescein was obtained from Molecular Probes, Inc. (Eugene, OR). Phospholipase C (type VIII, from *Bacillus cereus*) was purchased from Sigma Chemical Co. (St. Louis, MO).

Antibodies

A monoclonal antibody (mAb 143-3) against rat alkaline phosphatase that does not block the AP enzymatic activity and precipitates 80% of AP activity from ROS 17/2.8 cell extract was used in this study (Nair et al., 1987). The mAb 143-3 was purified from mouse ascites by protein A-affinity chromatography (Bio-Rad Laboratories, Richmond, CA). The Fab' fragments were generated by pepsin digestion and reduction (Perham, 1983; Cowan et al., 1986). Purified mAb 143-3 IgG (10 mg) was incubated for 1 h at 37°C with 250 μl of pepsin-coated agarose beads (Pierce Chemical Co., Rockford, IL) in 0.1 M citrate buffer (pH 3.5) in a total volume of 1 ml. The pH was neutralized with 1/10 vol of 0.3 M Tris (pH 8.6), and the pepsin-coated beads were removed by centrifugation. F(ab')₂ was reduced by addition of 1/5 vol of 100 mM cystein-HCl for 1 h at 37°C, and alkylated with 30 mM iodoacetamide for 15 min at room temperature. According to nonreducing SDS-polyacrylamide gels stained with Coomassie Blue, the Fab' fragments were completely free of contaminating material, except for some over-reduced 20-kD fragments. The second antibody was fluorescein isothiocyanate-conjugated Fab goat anti-mouse IgG (Cooper Biomedical, Malvern, PA).

Cell Preparation

Osteoblast-like cells, fraction 3 (F3), were obtained from 19-d fetal rat calvaria by collecting the cells released between 40–60 min enzymatic digestion (Wong and Cohn, 1974) in 2% collagenase and 1% hyaluronidase. The cells released during the first 20 min yielded the "fibroblast-rich" fraction, fraction 1 (F1). Fetal rat skin fibroblasts were obtained by an outgrowth culture of minced tissue from the same fetus, in Ham's F12 medium supplemented with 5% horse serum, 2% FBS, and 0.1 mg/ml kanamycin. At confluence, the cells were trypsinized and were plated into 4-well plates (Nunc) with 1 ml of above media. AP lateral mobility was measured 24–96 h later.

ROS 17/2.8 cells, a clonal osteoblastic cell line derived from a spontaneous rat osteosarcoma and ROS 25/1, nonosteoblastic cells derived from the same osteosarcoma (Majeska et al., 1980), were cultured in Ham's F12 medium supplemented with 5% FBS and 0.1 mg/ml kanamycin. Cells were subcultured (1:4) once a week and were prepared for experiments as de-

scribed for calvaria cells. UMR106 cells, a clonal osteoblastic cell line derived from a radiation-induced osteosarcoma (Patridge et al., 1980), were cultured in alpha-MEM medium supplemented with 10% FBS and 0.1 mg/ml kanamycin. Cells were subcultured (1:20) once a week and prepared for experiments as described above.

For hormonal treatments, ROS 17/2.8 cells were seeded at 10^4 cells/cm² as described above. Media were replaced 24 h later with fresh media containing dexamethasone (10^{-7} M) or $1,25(\text{OH})_2$ vitamin D₃ (10^{-9} M) in ethanol (0.1% vol/vol), or ethanol alone, and cells were cultured for 48–96 h before measurements.

To measure the lateral mobility of AP, cells were rinsed three times with Ham's F12 medium supplemented with 0.5% BSA followed by incubation with Fab' fragments of anti-rat AP mAb (19 $\mu\text{g}/\text{ml}$) for 7 min. After rinsing three times, cells were labeled with fluorescein-conjugated goat anti-mouse IgG Fab fragments (1:50 serum dilution) for 7 min. For lipid mobility, cells were labeled with hexadecanoyl aminofluorescein (HDAF) (7.5 ng/ml in Ham's F12 medium with 0.05% ethyl alcohol) for 5 min. After labeling, cells were rinsed five times with Ham's F12 medium supplemented with 0.5% BSA and twice with Ham's F12 medium without phenol red or vitamins. Cells were used immediately for the photobleaching measurements. All procedures were done at room temperature (23°C) unless stated otherwise. Control cell cultures were fixed in either methanol (at -70°C for 60 min), 2.5% glutaraldehyde (pH 7.4 at room temperature for 2 min), or 4% paraformaldehyde (at room temperature for 10 min) before antibody labeling.

Digestion with PI-PLC

Cells were seeded at indicated cell densities and were cultured on Lab-tek (Miles Scientific Laboratories, Naperville, IL) or in 96-well plates (Costar) for several days. Cells were rinsed twice with Ham's F12 medium and were treated with PI-PLC (20 $\mu\text{g}/\text{ml}$), kindly provided by Dr. Martin G. Low, or with 5 U/ml nonspecific phospholipase C (type VIII; Sigma Chemical Co.). Other cells were treated with vehicle only. Enzymatic digestion was carried out at 37°C for 60 min in Ham's F12 medium. No morphological change or cell detachment was observed during this treatment. After digestion, the supernatant enzyme solution was collected and cells were washed three times with Ham's F12 medium followed by extraction in 0.1% Triton buffer (10 mM Tris-HCl pH 7.4, 0.5 mM MgCl₂). AP enzymatic activity was determined in supernatant solution and in cell extracts by hydrolysis of paranitrophenyl phosphate (Majeska et al., 1985).

Fluorescence Redistribution after Photobleaching (FRAP)

Photobleaching experiments were conducted as previously described (Koppel, 1979; Koppel and Sheetz, 1983). Cells growing in Nunc wells were illuminated with laser light ($\lambda = 488 \text{ nm}$) masked to form a pattern of stripes. A Ronchi ruling (50 lines/in) is imaged onto the field diaphragm of the vertical illuminator of a Leitz Ortholux II microscope and reimaged onto the sample through a 50 \times , 1.00 NA water immersion objective. Illumination in this configuration, typically, covered a 60- μm -diam area with a pattern of stripes with a repeat distance of 5.0 μm . To minimize edge effects in the collected signal (Koppel and Sheetz, 1983), a diaphragm in the secondary image plane above the microscope limited the fluorescence observed to that from a 40- μm -diam area in the center of the illuminated region.

A brief pulse (typically 50 ms for measurements of protein diffusion, 10 ms for lipid diffusion) of intense laser light produces a periodic pattern of unbleached areas. Fluorescence redistribution is followed as a function of time after bleaching with a series of 12-point fluorescence scans with an attenuated laser pattern, produced by a galvanometric scanning mirror placed between the Ronchi ruling and the illuminator field diaphragm. Fluorescence intensity is detected by a cooled photomultiplier tube and quantitated with photon-counting electronics synchronized to the scanning laser pattern. For illustrative purposes, Fig. 1 A and B present the raw fluorescence scan data for AP on a transfected fetal rat skin cell.

Data Analysis

To correct for prebleach intensity variations, each postbleach fluorescence scan is normalized with a point-by-point division by the average of the prebleach scans. The Fourier modulation amplitude ($|A(\xi_0, t)|$) at the spatial frequency ($\xi_0 = 2\pi/d$ of the pattern repeat distance (d)) is calculated for each normalized scan (Koppel and Sheetz, 1983). For a single diffusing component, theory (Koppel and Sheetz, 1983) predicts an exponential decay of $|A(\xi_0, t)|$ with a decay rate $\Gamma = D\xi_0^2$. For a multicomponent system,

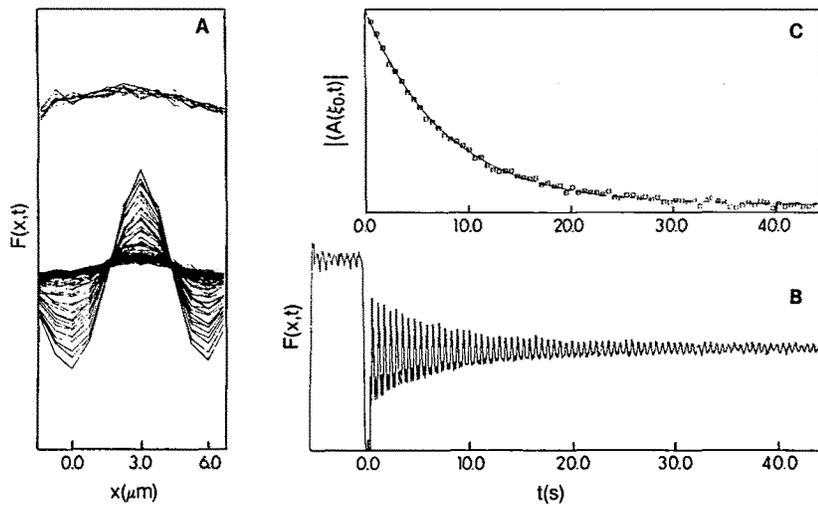


Figure 1. Fluorescence redistribution after photobleaching data for AP on a transfected fetal rat skin cell. (A) Superposition of eight prebleach (top) and 75 postbleach 12-point fluorescence scans. $F(x, t)$ is the total fluorescence intensity detected at time t with the monitoring illumination pattern shifted a distance x (along the scan axis orthogonal to the stripes) relative to the position of the bleaching pattern. (B) The same data set presented sequentially as a function of t . (C) $|A(\xi_0, t)|$, the amplitude of the fluorescence modulation at the fundamental spatial frequency (ξ_0), calculated for each postbleach scan. The solid line is the computed least-squares three-parameter fit to the data, giving a diffusion coefficient of $1.22 \times 10^{-9} \text{ cm}^2\text{s}^{-1}$ with 96% recovery.

one would have the corresponding sum or distribution of exponentials. The data were fitted by computer nonlinear least-squares analysis to a series of 2, 3, 4, and 5 parameter functions:

$$A_1^{(2)} \exp(-\Gamma_1^{(2)} t)$$

$$A_1^{(3)} \exp(-\Gamma_1^{(3)} t) + A_2^{(3)}$$

$$A_1^{(4)} \exp(-\Gamma_1^{(4)} t) + A_2^{(4)} \exp(-\Gamma_2^{(4)} t)$$

$$A_1^{(5)} \exp(-\Gamma_1^{(5)} t) + A_2^{(5)} \exp(-\Gamma_2^{(5)} t) + A_3^{(5)},$$

where the superscripts indicate the number of parameters. At each stage, the goodness of fit is analyzed with an F -test (Bevington, 1969) to check if an additional parameter is statistically justified.

The three parameter fit corresponds to the model usually invoked in presenting the results of diffusion measurements. A single diffusion coefficient,

$$D = (d/2 \pi)^2 \Gamma_1^{(3)},$$

characterizes the mobility of all labeled molecules free to diffuse on the time scale of the experiment. The percent of recovery after bleaching,

$$\%R = 100 \times A_1^{(3)} / (A_1^{(3)} + A_2^{(3)}),$$

corresponds to the fraction of mobile molecules. Fig. 1 C presents a plot of the postbleach modulation amplitudes, calculated for the data of Fig. 1, A and B. The solid curve is the computed least squares, three-parameter fit to the modulation decay.

Transient Expression of AP in Rat Cells

The AP expression vector used in this study was constructed by ligating a blunt ended 2,049-bp fragment of full-length rat osteosarcoma (ROS 17/2.8) AP cDNA, containing the coding region and a segment of the 5' and 3' untranslated region, into the Sma I site of plasmid vector, which contains SV40 early promoter, enhancer, and poly-linker (Ellis et al., 1986).

Transfection experiments were performed by a modification of the calcium precipitation procedure (Gorman, 1985). 1 μg of plasmid DNA was added as a calcium phosphate precipitate to a 2-cm² tissue culture well containing $2-4 \times 10^4$ cells that had been plated 24 h earlier in 1 ml medium. A precipitate was produced by dropping a mixture of 1 μg DNA in 1 μl , 6.2 μl 2 mM CaCl_2 , and 42.8 μl water into 50 μl of double strength Hepes-buffered saline (HBS) which contained 1.636% NaCl (wt/vol), 1.188% Hepes (wt/vol), 0.04% Na_2HPO_4 (wt/vol). Then the solution containing precipitate was transferred into each well. After a 3-h incubation, medium was replaced by 0.1 ml of 15% glycerol/HBS and cells were incubated for 3 min at 37°C. Cells were then washed, were fed with complete media, and were cultured for 48-72 h before measurements.

Results and Discussion

Lateral Mobility of Endogenous AP and of Lipid Probe in Osteoblast-like Cells

The AP diffusion coefficients (D) in UMR106 and osteoblastic (F3) fetal rat calvaria cells were $(1.77 \pm 0.51) \times 10^{-9} \text{ cm}^2\text{s}^{-1}$ and $(1.28 \pm 0.35) \times 10^{-9} \text{ cm}^2\text{s}^{-1}$ respectively (Table I a; Fig. 2), 6-10 times faster than D for many membrane proteins ($\sim 2 \times 10^{-10} \text{ cm}^2\text{s}^{-1}$). The percent recoveries (%R) of AP in these cells were 82.8 ± 2.5 and 73.3 ± 4.0 , respectively (Table I a), values that are larger than those of most membrane proteins. The diffusion coefficient of AP in ROS 17/2.8 cells was $(0.58 \pm 0.16) \times 10^{-9} \text{ cm}^2\text{s}^{-1}$ (Table I a; Fig. 2). This value is still faster than that of other membrane proteins but two to threefold slower than that of UMR106 and F3 cells, indicating that D for AP is somewhat dependent on cell type. The mobile fraction of AP in ROS 17/2.8 cells was $73.3 \pm 10.5\%$, similar to that in other bone cell types (Table I a). These results, of course, represent the diffusion characteristics of the tagged alkaline phosphatase, which includes the Fab fragments in addition to the membrane bound protein.

No recovery from photobleaching was observed on cells fixed with methanol or glutaraldehyde before antibody labeling. This verifies that the rapid fluorescence redistribution observed in unfixed cells corresponds to surface antigen mobility rather than reflecting the rate of binding and release of the antibody marker. Paraformaldehyde fixation also decreased the rate and extent of redistribution, but did not eliminate it altogether.

The diffusion coefficients of lipid molecules, estimated by the fluorescent lipid probe HDAF, were $(5.03 \pm 0.53) \times 10^{-9} \text{ cm}^2\text{s}^{-1}$ in F3 calvaria cells and $(4.28 \pm 0.51) \times 10^{-9} \text{ cm}^2\text{s}^{-1}$ in ROS 17/2.8 cells at 23°C (Table II a; Fig. 2). Although the HDAF probe contains only a single hydrophobic hydrocarbon tail, model membrane studies (Derzko and Jacobson, 1980) indicate that it should provide at least a useful relative measure of the lateral self-diffusion coefficient of membrane phospholipid. These findings show: (a) that AP mobility, at its fastest, is closer to lipid mobility than to that of other reported membrane proteins, though still slightly re-

Table I. Lateral Mobility of AP on Plasma Membranes of Osteoblast-like Cells

Cells	Treatment	$D \times 10^9$ $cm^2 s^{-1}$	%R	n
<i>a</i>				
UMR106		$1.77 \pm 0.51^*$	$82.8 \pm 2.5^\S$	17
F3		$1.28 \pm 0.35^\ddagger$	$73.3 \pm 4.0^\S$	3
ROS 17/2.8		$0.58 \pm 0.16^{*\ddagger}$	73.3 ± 10.5	21
<i>b</i>				
Fetal rat skin	TRF	$1.06 \pm 0.22^*$	86.7 ± 9.4	9
Fetal rat calvaria (F1)	TRF	$1.13 \pm 0.30^\ddagger$	75.7 ± 7.6	6
ROS 25/1	TRF	0.92 ± 0.31	80.7 ± 8.8	7
Fetal rat calvaria (F3)	TRF	$1.70 \pm 0.25^\S$	89.3 ± 2.1	7
ROS 17/2.8	D-TRF	$0.68 \pm 0.09^{*\ddagger}$	69.3 ± 4.8	4
<i>c</i>				
ROS 17/2.8	Control	$0.58 \pm 0.13^{*\ddagger}$	71.3 ± 10.0	12
ROS 17/2.8	Dexamethasone (10^{-7} M)	$0.80 \pm 0.16^*$	79.6 ± 6.1	14
ROS 17/2.8	1,25(OH) $_2$ D $_3$ (10^{-9} M)	$0.80 \pm 0.22^\ddagger$	67.4 ± 9.4	5
<i>d</i>				
ROS 17/2.8	10×10^3 cells/cm 2	0.55 ± 0.13	70.5 ± 13.6	6
ROS 17/2.8	80×10^3 cells/cm 2	0.63 ± 0.16	72.1 ± 8.1	12

(a) UMR106, F3 (fraction 3) fetal rat calvaria, and ROS 17/2.8 cells were plated at $1-8 \times 10^4$ cells/cm 2 in 2 cm 2 wells 1-5 d before the FRAP measurements. Data are shown as mean \pm SD in *a-d*. D , diffusion coefficients; %R, percent recoveries; n , number of measurements. *, \ddagger , \S , $p < 0.01$, for Student *t*-test of two-tailed hypothesis, relative to the group identified by the same symbol. (b) Cells were seeded at 1×10^4 cells/cm 2 followed by transfection or mock transfection 24 h later. The cells were then cultured for 48-96 h before FRAP measurements. TRF, transfected with rat AP cDNA; D-TRF, dummy transfection without rat AP cDNA. *, $p < 0.01$; \ddagger , $p < 0.05$; \S , $p < 0.001$. (c) ROS 17/2.8 cells were plated at 1×10^4 cells/cm 2 followed by replacement with fresh media containing either hormones or vehicle (ethanol 0.1% vol/vol) 24 h after seeding. Cells were then cultured for 48-96 h before FRAP measurements. *, $p < 0.01$; \ddagger , $p < 0.05$. (d) ROS 17/2.8 cells were seeded at the indicated cell density 48 h before FRAP measurements.

stricted compared to lipid, and (b) that there is no dramatic difference in the lipid diffusion coefficients between F3 cells and ROS 17/2.8 cells which could account for the differences in AP diffusion. The mobile fractions of HDAF were also similar, $83.6 \pm 3.2\%$ and $74.8 \pm 6.6\%$, respectively (Table II *a*).

Our analysis of the fluorescence data assumes diffusion in a plane. For curved or ruffled cellular surfaces, the calculated values of D should be considered as the lower boundary of the true in-plane diffusion coefficients. In any event, the effect of membrane geometry would not change the relative values of D , when protein diffusion in the membrane is compared with lipid diffusion.

Our measurements of lipid diffusion indicate that the slower AP diffusion in ROS 17/2.8 cells is not due to differences in membrane geometry, the viscosity of the lipid bilayer or membrane protein density (Vaz et al., 1984). The factors that could limit the lateral diffusion of AP in these cells include interactions mediated by PI where AP is anchored, and interactions with extracellular components (glycocalyx). A high AP concentration in plasma membrane is not a likely cause for the slower diffusion in ROS 17/2.8 cells since identical diffusion coefficients were obtained in ROS 17/2.8 cells which varied by a factor of two in AP fluorescence (data not shown), and AP levels estimated by activity or fluorescence staining in UMR106 cells (Patridge et al., 1980) or transfected cells were similar to those in ROS 17/2.8 cells.

The slower diffusion of AP in ROS 17/2.8 cells could result from differences in the glycocalyx or differences in the extent of AP glycosylation. The same gene is believed to code for bone, liver, kidney, and placenta AP in the rat (Wilcox and Taylor, 1981), but there are differences in posttranslational

glycosylation. Prior comparison of AP from several rat tissues suggested more extensive glycosylation of ROS 17/2.8 AP, which had an apparent subunit molecular mass of $\sim 82,000$ D, vs. 75,000 D for bone and kidney and 90,000 D for placenta, all of which converted to 52,000 D after degly-

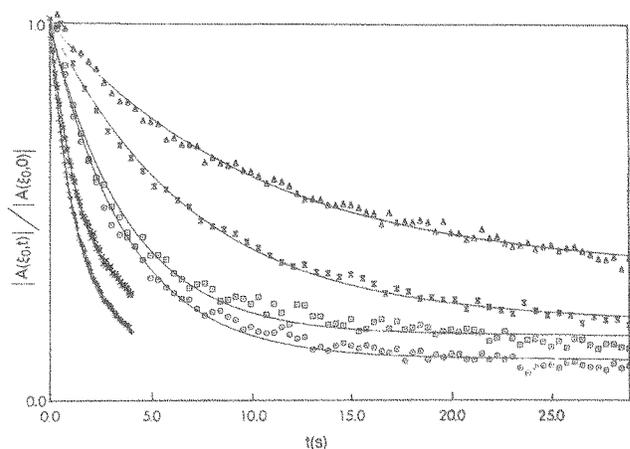


Figure 2. Representative traces of fluorescence modulation amplitudes. Each tracing is the average of amplitudes from 7 to 16 individual sets of data. From the slowest to the fastest, they represent: AP on ROS 17/2.8 cells (Δ), AP on ROS 17/2.8 cells treated with dexamethasone (\times), AP on UMR106 cells (\square), AP on transfected fraction 3 cells (\circ), HDAF on ROS 17/2.8 cells (\times), and HDAF on fraction 3 cells ($+$). All experiments except the HDAF data collection, were performed with a periodic pattern repeat distance (d) of 5.0 μ m. For HDAF, d equals 6.67 μ m; but the time axis has been scaled by a factor of $(5.0/6.67)^2$ for direct comparison to the rest of the data. The solid curves are the computed least-squares three-parameter fits to the data.

Table II. Lateral Mobility of HDAF in Plasma Membrane of Osteoblast-like Cells

Cells	Treatment	$D \times 10^9$ cm^2s^{-1}	%R	n
a				
F3	23°C	5.03 ± 0.53	83.6 ± 3.2*	5
ROS 17/2.8	23°C	4.28 ± 0.51	74.8 ± 6.6*	5
b				
ROS 17/2.8	34°C			
	Control	16.4 ± 1.7	79.4 ± 2.3	5
	Dexamethasone (10^{-7} M)	14.5 ± 1.6	77.3 ± 2.2	5
F3	34°C			
	Control	19.4 ± 1.5	90.3 ± 3.0	10
	Dexamethasone (10^{-7} M)	21.7 ± 2.3	90.8 ± 1.5	9

(a) F3 and ROS 17/2.8 cells were plated at $2-5 \times 10^4$ cells/cm² 24 h before the FRAP measurements. Measurements were taken at room temperature (23°C). Data are shown as mean ± SD, as in b. *D*, diffusion coefficients; %R, percent recoveries; *n*, number of measurements. *, $p < 0.05$, for Student *t*-test of two-tailed hypothesis. (b) ROS 17/2.8 and F3 cells were plated at $2-5 \times 10^4$ cells/cm² 24 h before FRAP measurements, which were conducted at 34°C, where previous dexamethasone effects were reported.

cosylation (Nair et al., 1987). On the other hand, the apparent molecular mass of AP in UMR106 is 78,000–80,000 D (Hsu, H. H. T., personal communication). The effect of glycosylation on mobility has been shown directly by Wier and Edidin (Wier, M. L., and M. Edidin, personal communication), who showed that the diffusion coefficients of major histocompatibility antigen increased severalfold after genetic truncation of glycosylation sites (Miyazaki et al., 1986). Similar experiments should now be possible on AP.

It is of interest that when divalent mAbs 143-3 were used in conjunction with Fab second antibody, AP mobility (*D*) in ROS 17/2.8 cells was reduced by 50%, probably due to bridging between AP molecules, forming dimers (McComb, 1979) or tetramers (Chakrabaratty and Stinson, 1985) in the membrane. However, these multimeric form should not affect the measurement of *D* in this study, since Fab monovalent fragments were used both as first and second antibodies.

Lateral Mobility of Transiently Expressed AP

To examine if the AP diffusion properties were intrinsic to the AP molecule or were related to the host cell, rat AP cDNA from ROS 17/2.8 cells were transiently expressed in several cell types. The transfection procedure itself, without addition of DNA, did not alter the AP diffusion coefficient [$D = (0.68 \pm 0.09) \times 10^{-9}$ cm²s⁻¹] or the mobile fraction (%R = 69.3 ± 4.8) in ROS 17/2.8 cells (Table I b).

The diffusion coefficient and mobile fraction of AP tran-

siently expressed in fetal rat skin fibroblasts were $(1.06 \pm 0.22) \times 10^{-9}$ cm²s⁻¹ and 86.7 ± 9.4%, respectively (Table I b), values comparable with those of endogenously expressed enzyme (Table I a; Fig. 2). Transient expression of AP in the nonosteoblastic osteosarcoma cell line (ROS 25/1), which had undetectable AP levels before transfection, yielded a diffusion coefficient of $(0.92 \pm 0.31) \times 10^{-9}$ cm²s⁻¹, which was slower than in UMR106 ($p < 0.001$), suggesting that this is a property of the ROS type cells (Table I b). AP expressed in calvaria-derived F3 cells, identified by high levels of AP activity, had $D = (1.70 \pm 0.25) \times 10^{-9}$ cm²s⁻¹ and %R = 89.3 ± 2.1, not significantly different from the AP natively expressed (Table I b; Fig. 2). These findings suggest that the relatively faster diffusion of AP is a property of the AP molecule and that the small differences among cell types may be due to posttranslational modifications.

Effect of Phosphatidylinositol-specific Phospholipase C (PI-PLC) on Endogenous and Transfected AP

PI-PLC released >80% of AP activity from UMR106 and ROS 17/2.8 (Tables III and IV), 75% from transfected ROS 25/1, and 60% from transfected skin cells (Table IV). Control medium or nonspecific phospholipase C did not significantly release AP from these cells (Table IV). These findings indicate that AP is anchored in the membrane via a phosphatidylinositol linkage in osteoblast-like cells and in transfected cells. Therefore the transfected AP-negative cells must contain the machinery needed to transfer and modify the translation product of ROS 17/2.8 AP cDNA and link it to PI. Since AP, 5'-nucleotidase, and Thy-1 antigen are often expressed in the same cells, the processing that links these proteins to PI may be similar. The posttranslational modifications and intracellular transport of variant surface glycoprotein in *Trypanosoma* have been recently described (Bangs et al., 1986), and further studies are needed to establish the generality of the glycopospholipid anchor for PI-PLC-released molecules and the function of this membrane attachment.

Effect of Dexamethasone, 1,25(OH)₂D₃, and Cell Density on the Lateral Mobility of AP and of the Lipid Probe in ROS 17/2.8 Cells

Glucocorticoids and vitamin D have pronounced effects on alkaline phosphatase expression and on the shape of ROS

Table III. Release of AP Activity by Phosphatidylinositol-specific Phospholipase C

Cells	PI-PLC concentration μg/ml	AP activity ± SD		n	R
		Cell nmol/min/well	Supernatant nmol/min/well		
ROS 17/2.8	0	22.6 ± 3.6	0.7 ± 0.2	3	0.03
	2	18.2 ± 1.5	2.8 ± 0.9	3	0.13
	20	2.1 ± 0.3	19.8 ± 4.1	3	0.90
UMR106	0	35.5 ± 0.6	0.3 ± 0.1	3	0.01
	2	21.3 ± 0.6	20.5 ± 0.7	3	0.49
	20	5.5 ± 1.6	36.1 ± 1.9	3	0.87

Cells were plated at 1×10^4 cells/cm² and were cultured for 48 h before treatment with PI-PLC. SD, standard deviation; *n*, number of wells; *R*, fraction of released AP activity into medium.

Table IV. Effect of Phospholipase C and Phosphatidylinositol-specific PLC on the Release of Native and Transfected AP

Cells	AP activity released into medium		
	Control	Nonspecific PLC	PI-specific PLC
	%	%	%
UMR106	0.4 ± 0.2 (5)	0.3 ± 0.1 (5)	91.4 ± 2.8 (5)
ROS 17/2.8	0.3 ± 0.1 (5)	1.3 ± 0.4 (5)	91.3 ± 1.2 (5)
ROS 25/1 (transfected)	9.2 ± 0.3 (5)	9.0 ± 1.9 (5)	75.1 ± 7.5 (5)
Skin (transfected)	2.3 ± 0.7 (5)	2.5 ± 0.8 (5)	60.1 ± 4.0 (5)

ROS 17/2.8 and UMR106 cells were plated at 5×10^4 cells/cm² 48 h before treatment with PLC or PI-PLC. ROS 25/1 and skin cells were plated at 5×10^4 cells/cm² 24 h before transfection followed by subsequent culture for 48 h before treatment with the enzymes. Released fraction of AP activity (%) is defined as $S/(C+S) \times 100$, where C and S are the AP activity per well in cells and supernatant, respectively. Data are shown as mean ± SD. Numbers in parentheses indicate numbers of wells.

17/2.8 cells, the latter, presumably, through cytoskeletal involvement. Glucocorticoids increase the level of AP several-fold (Majeska et al., 1985) and cause cell spreading and actin cable formation (Wiren and Rodan, 1985). The active metabolite of vitamin D, 1,25(OH)₂D₃, causes stellar shape changes followed by cell rounding and decreases the length of actin filaments (Gronowicz et al., 1986). We examined the effects of glucocorticoids (10^{-7} M) and 1,25(OH)₂D₃ (10^{-9} M) on AP mobility, and found that both hormones increased D by ~40% ($p < 0.001$; Table I c; Fig. 1), but had no significant effect on the mobile fraction. Although dexamethasone reduces cholesterol synthesis (Johnston et al., 1980) and therefore increases lipid mobility in HeLa cells (Boullier, 1982) and lymphocytes (Keating et al., 1985), it did not in this study where the lipid mobility and mobile fraction measured at 34°C remained virtually unchanged by dexamethasone treatment (Table II b). Thus, neither effects on lipid metabolism nor on the microfilament cytoskeleton, affected differently by the two hormones, explain the rise in AP mobility. Both hormones affect the differentiation of these cells (Rodan and Rodan, 1984) and future insights into their action may further clarify their common effect on AP mobility.

High cell density was reported to restrict the lateral mobility of MHC antigen in fibroblast cultures (Wier and Edidin, 1986), probably by modifying the interaction of this molecule with extracellular matrix. Cell density affects AP levels in ROS 17/2.8 cells (Noda et al., 1986) but had no effect on AP mobility: the same D and % R were measured in cells plated at 10^4 and 8×10^4 cells/cm² (Table I d).

Conclusions

PI-PLC has been shown to release 60–90% of native and transfection-expressed AP in a variety of osteoblastic and nonosteoblastic embryonic and tumor cells. This demonstrates that AP is anchored in the membrane of these cells by linkage to PI, indicating that even those cells that do not normally express the protein possess the machinery for PI anchorage. AP in all cell types showed rapid lateral diffusion (similar to that shown for Thy-1, another PI-linked surface protein), with near complete fluorescence recovery. At its fastest, AP diffusion rates are closer to those shown for membrane lipids than those of most conventionally anchored integral membrane proteins. There is some evidence of a dependence of diffusion rates on cell type, but it is not known at this point if the relatively small variations observed arise

from differences in cell glycocalyx, posttranslational AP glycosylation, or some other factors. Taken together, these results argue against the idea that interaction with the extracellular glycocalyx by itself accounts for the markedly reduced lateral diffusion rates observed with other integral membrane proteins, and point to the important role that protein–protein interactions within the bilayer may play in this process.

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