A Chinese Hamster Ovary Cell Mutant Defective in the Non-endocytic Uptake of Fluorescent Analogs of Phosphatidylserine: Isolation Using a Cytosol Acidification Protocol

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Abstract. Transmembrane movement of phosphatidylserine (PS) and various PS analogs at the plasma membrane is thought to occur by an ATP-dependent, protein-mediated process. To isolate mutant CHO cells defective in this activity, we first obtained conditions which inhibited the endocytic, but not the non-endocytic pathway of lipid internalization since PS may enter cells by a combination of these two pathways. We found that acidic treatment of cells, which blocks clathrin-dependent endocytosis, enhanced the energy-dependent uptake of 1-palmitoyl-2-(6-[{7-nitrobenz-2-oxa-1,3-diazol-4-yl}amino]caproyl-*sn*-glycero-3-phosphoserine (C_6-NBD-PS) in CHO cells from donor vesicles (liposomes) by about twofold. Control experiments demonstrated that the enhanced uptake of C_6-NBD-PS at acidic pH was not due to: (*a*) an increase in the capacity of the plasma membrane to incorporate C_6-NBD-PS from the donor vesicles; (*b*) a decrease in the rate of loss of C_6-NBD-PS from the cells; or (*c*) fusion or engulfment of the donor vesicles. When cytosolic acidification (to pH 6.3) was imposed without acidification of the extracellular medium, C_6-NBD-PS uptake by intact cells was increased by about 50% compared to control values determined in the absence of acidification. These results suggested that a protein and energy dependent system(s) for transbilayer movement of the fluorescent PS was stimulated by cytosolic acidification.

A screening method for mutant cells defective in the non-endocytic uptake of fluorescent PS analogs with replica cell colonies at acidic pH was then devised. After selection of mutagenized CHO-K1 cells by in situ screening, we obtained a mutant cell line in which uptake of fluorescent PS analogs was reduced to about 25% of the wild type level at either pH 6.0 or 7.4. Control experiments demonstrated that the reduced uptake of fluorescent PS analogs in the mutant cells was unrelated to multidrug resistance, and that endocytosis of another plasma membrane lipid marker occurred normally in the mutant cells. These results suggested that a non-endocytic pathway responsible for uptake of fluorescent PS analogs was specifically affected in the mutant cells.

THE membranes of all mammalian cells contain numerous classes of glycerolipids and sphingolipids. However, these molecules are not randomly distributed in all intracellular membranes, but rather, different organelles have different lipid compositions (reviewed in White, 1973; Voelker, 1991). Furthermore, an asymmetric distribution of lipid types across the membrane bilayer is sometimes observed, particularly in the plasma membrane where the choline-containing phospholipids, phosphatidylcholine, and sphingomyelin, are highly enriched on the exoplasmic leaflet of the membrane bilayer while the aminophospholipids such as phosphatidylserine (PS)[1] and phosphatidylethanolamine are enriched on the cytoplasmic leaflet (reviewed in Schroit and Zwaal, 1991; Devaux, 1992; Zachowski, 1993). Although the physiological significance of lipid asymmetry is still unclear, a number of plausible roles for lipid asymmetry have been suggested. For example, in model membrane systems it has been shown that PS or phosphatidylethanolamine

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1. *Abbreviations used in this paper*: ABS, acetate-buffered saline containing 20 mM Na acetate/acetic acid, 137 mM NaCl, 2.7 mM KCl, 0.32 mM Na_2HPO_4, 1.3 mM $CaCl_2$, 0.8 mM $MgSO_4$ and 5.5 mM D-glucose; C_6- or C_{12}-NBD, 6- or 12-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl or dodecanoyl; C_6-NBD-PS, 1-palmitoyl-2-C6-NBD-*sn*-glycero-3-phosphoserine; 1-C_6-2-C_{12}-NBD-PS, 1-hexanoyl-2-C_{12}-NBD-*sn*-glycero-3-phosphoserine; C_6-NBD-SM, *N*-C_6-NBD-sphingosylphosphorylcholine; DOPC, dioleoyl phosphatidylcholine; HBS, Hepes-buffered saline containing 20 mM Hepes/NaOH, 137 mM NaCl, 2.7 mM KCl, 0.32 mM Na_2HPO_4, 1.3 mM $CaCl_2$, 0.8 mM $mgSO_4$ and 5.5 mM D-glucose; HMEM, Hepes-buffered MEM; PS, phosphatidylserine.

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are fusogenic (reviewed in Düzgünes et al., 1987). Thus, the preferential distribution of these phospholipids at the cytoplasmic face of membranes may be important in promoting intracellular fusion events during transport of intracellular vesicles, and in preventing unnecessary fusion between cells. The asymmetric distribution of PS at the plasma membrane may also be relevant to activation of protein kinase C which plays a central role in signal transduction (Nishizuka, 1992). In addition, it is interesting to note that when certain cell types lose their asymmetric distribution of PS and that lipid is exposed on the cell surface, they are efficiently cleared by macrophages, suggesting that cell surface PS molecules may serve as one of the markers of dying cells (Savill et al., 1993). It has been demonstrated that PS exposure on membranes accelerates formation of tenase and prothrombinase complexes, thereby activating the coagulation cascade in blood clotting processes (Schroit and Zwaal, 1991; Esmon, 1993). These examples indicate that the regulation of lipid asymmetry may have significant physiological consequences and thus represents an important problem in cell and membrane biology.

In artificially generated lipid vesicles the transbilayer movement ("flip/flop") of charged phospholipids is very slow ($t_{1/2}$ ≈hours to days; reviewed in Dawidowicz, 1987), presumably because transport of the charged head group through the hydrophobic core of the membrane bilayer represents a high energy barrier. By contrast, in biological membranes, certain phospholipids can undergo rapid transbilayer movement, suggesting the existence of a facilitated transport mechanism for this process (reviewed in Voelker, 1991; Devaux, 192; Zachowski, 1993). Indeed, a protein-mediated transport for PS was originally proposed to explain the inward transbilayer movement of a spin-labeled PS analog in human erythrocytes which could be inhibited by ATP depletion (Seigneuret and Devaux, 1987). Subsequent reports documented the facilitated transbilayer movement of PS or other analogs, in various cell types (reviewed in Schroit and Zwaal, 1991; Devaux, 1992; Zachowski, 1993). The ATP-dependent PS transport system is unlikely to be a simple passive transporter facilitating an equal distribution of PS between both leaflets of the plasma membrane bilayer, but rather seems to mediate the active accumulation of PS from the exoplasmic leaflet to the cytoplasmic one. It is thought that this transport system is involved in the maintenance of PS asymmetry.

Although several recent reports have suggested the identification of the PS transporter (Morrot et al., 1990; Schroit et al., 1990; Zimmerman and Daleke, 1993), the molecular characteristics of the PS translocating system are not yet understood, and, in particular, little is known about the physiological factors which regulate the transporter. In addition, although a genetic approach would be useful for investigating PS transport, to date there are no reports on mutant cells defective in PS transport. Since plasma membrane PS can enter cells in principle by a combination of endocytic and non-endocytic pathways, conditions in which the former is inhibited are required when one wants to select mutant cells defective in the non-endocytic pathway. In the present study, we examined the effects of pH on the uptake of 1-palmitoyl-2-C_6-NBD-*sn*-glycero-3-phosphoserine (C_6-NBD-PS), a well-characterized probe for the PS-translocating system, because treatment of the cells at low pH blocks clathrin-dependent endocytosis (Davoust et al., 1987; Sandvig et al., 1987; Heuser, 1989). We found that acidification of the cytosol stimulated C_6-NBD-PS uptake in CHO-K1 fibroblasts and used this result as a basis for developing a screening method for isolation of CHO mutants defective in the non-endocytic uptake of fluorescent PS analogs.

Materials and Methods

Cell Culture

CHO-K1 fibroblasts (ATCC CCL 61; American Type Culture Collection, Rockville, MD) were cultured in Ham's F-12 medium (Biofluids Inc., Rockville, MD) supplemented with 2 mM L-glutamine 5% fetal bovine serum, penicillin G (100 U/ml), and streptomycin sulfate (100 μg/ml). For biochemical studies, 8×10^5 cells in 5 ml of medium were seeded into each 60-mm-diam tissue culture dish and grown for 24 h. For fluorescence microscopy, cells were grown on 25-mm-diameter glass coverslips (No. 1 thickness), placed inside of 35-mm-diam tissue culture dishes. For microscopy, cells were grown to ≈50-75% confluency. For measurements of cytosolic pH, 5×10^5 cells in 2 ml of culture medium were seeded into each 35-mm-diam culture dish containing a rectangular (4 × 9 mm) glass coverslip and cultured for 24 h. All cells were grown at 37°C in a water-saturated atmosphere of 5% CO_2 in air. However, when UPS-1 mutant cells were characterized, the mutant and the control cells were routinely maintained at 33°C. A multidrug-resistant CHO cell variant, ALLN^{r50} cell line (Sharma et al., 1992) was kindly provided by Dr. Robert Simoni (Department of Biological Sciences, Stanford University, Palo Alto, CA). The sensitivity of CHO cells to colchicine was examined as described previously (Rosenwald and Pagano, 1994).

Lipids and Miscellaneous Reagents

C_6-NBD-SM was from Molecular Probes, Inc. (Eugene, OR). Other NBD-phospholipids and dioleoyl phosphatidylcholine (DOPC) were from Avanti Polar Lipids, Inc. (Alabaster, AL). All lipids were stored in $CHCl_3$ at −20°C. NaN_3, 2-deoxy-D-glucose, NEM, amiloride, and colchicine were purchased from Sigma Chemical Co. (St. Louis, MO); nigericin was from Calbiochem-Novabiochem Corp. (La Jolla, CA).

Preparation of Fluorescent Phospholipid Donor Vesicles

Small unilamellar vesicles containing C_6-NBD-PS/DOPC (1:2, mol/mol), (or C_6-NBD-PS/C_6-NBD-sphingosylphosphorylcholine[SM]/DOPC [1:1:2, mol/mol/mol]) were typically prepared by ethanol injection (Kremer et al., 1977) as follows. Aliquots of the appropriate lipid stock solutions were mixed in a glass tube and dried under N_2. The dried lipids (0.6 μmol total lipid [0.8 μmol for DOPC/C_6-NBD-PS/C_6-NBD-SM]) were then dissolved in 50 μl absolute ethanol, and this solution was injected into 5 ml of deionized water while vortex mixing. An equal volume of a twofold concentrated saline solution at the appropriate pH value was then added. The final concentration of C_6-NBD-PS in the uptake buffer was 20 μM.

Incubation of C_6-NBD-Phospholipids with Cells and Analysis of Fluorescent Lipids

Unless noted otherwise, all manipulations were performed at 37°C. Monolayers cultures were rinsed twice with 2 ml of HBS (pH 7.4) or ABS (pH 6.0), and then further incubated in 2 ml of the same buffer for 5 min. The cells were then incubated with 1 ml of donor vesicles (20 μM C_6-NBD-PS/ml) in HBS (pH 7.4) or ABS (pH 6.0) for 10 min. The monolayer was then rinsed with 1 ml of ice-cold defatted 2% BSA/Hepes-buffered MEM (HMEM) and then incubated (3 × 10 min) with 2 ml of defatted 2% BSA/HMEM at 4°C to remove C_6-NBD-PS from the cell surface (referred to as "back-exchange;" Mohandas et al., 1982; Martin and Pagano, 1987; van Meer et al., 1987). The cells were then rinsed three times with 4 ml of ice-cold PBS, and harvested in 2.5 ml of PBS by scraping. The cells were collected by centrifugation (300 *g*, 5 min at 4°C), washed with 3 ml of PBS, and resuspended in 900 μl of ice-cold PBS. Aliquots were removed for protein content determination (Lowry et al., 1951), using BSA as a standard and for lipid extraction (Bligh and Dyer, 1959), using 0.9% NaCl and 10 mM HCl in the aqueous phase. The relative fluorescence of the lipid extracts was measured using an SLM-8000C spectrophotofluorometer (SLM/

Aminco, Urbana, IL) and the absolute amounts of fluorescent lipid were determined from calibration curves generated from known amounts of C_6-NBD-PS.

In experiments using lipid vesicles containing both C_6-NBD-PS and -SM, lipid extracts were analyzed by TLC on silica gel 60 plates (E. Merck, Darmstadt, FRG) using $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (90:40:12:2, vol/vol/vol/vol) as the developing solvent. The chromatograms were observed under UV illumination and the individual fluorescent lipids were quantified by image processing (Koval and Pagano, 1989).

ATP Depletion and NEM Treatment of Cell Monolayers

To deplete intracellular ATP, glucose-free ABS (pH 6.0) and HBS (pH 7.4) containing 5 mM NaN_3 and 50 mM 2-deoxy-D-glucose), designated ABS^{+inhib} and HBS^{+inhib}, respectively, were used. Monolayer cultures were preincubated in ABS^{+inhib} or HBS^{+inhib} for 5 min at 37°C, after which incubations were performed with 20 μM C_6-NBD-PS donor vesicles in ABS^{+inhib} or HBS^{+inhib}. To determine cellular ATP levels CHO monolayers incubated in various media for 10 min at 37°C were harvested in ice-cold PBS by scraping, and the ATP levels in aliquots of the cell suspension (~1 μg protein) were determined using a luciferin-luciferase assay kit (Technical Bulletin No. BSCA-1; Sigma Chemical Co., St. Louis, MO), except that a scintillation counter (Phillippy, 1994) was used in place of a bioluminometer.

To examine the effects of NEM treatment on C_6-NBD-PS uptake, cells were incubated with 2 ml of HMEM containing 0.2 mM NEM for 30 min at 4°C, and subsequently incubated with 2 ml of HMEM containing 0.2 mM dithiothreitol for 5 min at 4°C to quench unreacted NEM. The NEM-treated cells were then rinsed twice with ABS (pH 6.0) or HBS (pH 7.4), incubated in the same medium for 5 min at 37°C, and the uptake of C_6-NBD-PS was determined as described above.

Fluorescence Microscopy

Cells were incubated with fluorescent lipids as described above, except that the volume of the solutions used was reduced by one half. After back exchange, the cells were rinsed three times with 1 ml of ice-cold HMEM and observed and photographed under the fluorescence microscope. Microscopy was performed with a Zeiss IM-35 inverted microscope equipped with a Planapo 100× (1.3 NA) objective and a filter pack appropriate for NBD fluorescence. All exposures were 4 s using Kodak Tri-X film which was processed at ASA 1600 with Diafine developer.

Monitoring Cytosolic pH

Cytosolic pH was monitored with BCECF, a pH-sensitive fluorescent dye (Rink et al., 1982; Negulescu and Machen, 1990). All manipulations were at 37°C unless noted otherwise. Cell monolayers were rinsed with 1 ml of serum-free F-12 medium and then incubated in the dark for 15 min at room temperature with 2 ml of serum-free F-12 medium containing 4 μM BCECF-acetoxylmethyl ester (Molecular Probes Inc., Eugene, OR). The latter was prepared from a 2 mM BCECF acetoxmethyl ester/dimethylsulfoxide solution. The cells were then rinsed twice with 1 ml of serum-free F-12 medium, and then further incubated with 2 ml of serum-free F-12 medium for 15 min at room temperature in the dark to generate intracellular BCECF from the BCECF acetoxymethyl ester.

When cytosol acidification was imposed by the NH_3 load and release method (Boron, 1983) the BCECF-loaded monolayers were rinsed with 1 ml of HBS (pH 7.4) containing 30 mM NH_4Cl and incubated in 1 ml of the same buffer for 5 min. Then, after attachment to a positioning device (Di Virgilio et al., 1988), the cover slip was transferred to a cuvette containing 2.2 ml of 30 mM NH_4Cl in HBS (pH 7.4) and fluorescence intensity (λ_{ex} = 490 nm; λ_{em} = 542 nm) of the monolayer was monitored with a spectrofluorometer. To impose an NH_3 gradient, the coverslip with holder was transferred to another cuvette containing "exchange medium" (2.2 ml HBS, pH 7.4, in the presence or absence of 1 mM amiloride), and the monitoring was resumed. In some cases, 30 mM NH_4Cl was also added to the exchange medium so as not to impose an NH_3 gradient. For calibration of the cytosolic pH, the BCECF-loaded monolayer was incubated in 20 mM sodium phosphate buffer containing 50 mM KCl, 0.8 mM $MgSO_4$, 0.1% glucose, and 10 μM nigericin at various pH values. Intracellular BCECF was normalized by measurement of fluorescence intensity at pH-insensitive wavelengths (λ_{ex} = 439 nm; λ_{em} = 542 nm).

Isolation of CHO Mutant Cell Line, UPS-1

CHO-K1 cells were mutagenized with ethyl methanesulfonate and replica colonies of the cells on polyester disks were formed at 33°C as described previously (Hanada et al., 1990; Raetz et al., 1982). After incubation of replica disks at 39°C for 1 d in 5 ml modified F-12 containing 5% fetal bovine serum, the disks were washed three times with 4 ml ABS and then incubated in 4 ml ABS containing 20 μM 1-C_6-2-(12-[7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]dodecanoyl-*sn*-glycero-3-phosphoserine (1-C_6-2-(C_{12}-NBD)-PS)/40 μM DOPC for 1 h at 37°C. The disks were then transferred to dishes containing 10 ml of ice-cold PBS containing 50 mM $Na_2S_2O_4$, 1.3 mM $CaCl_2$, and 0.8 mM $MgSO_4$ for 30 s and washed four times with 5 ml of ice-cold PBS containing 1.3 mM $CaCl_2$ and 0.8 mM $MgSO_4$. This procedure destroys extracellular and cell surface (but not intracellular) NBD-fluorescence (McIntyre and Sleight, 1991). After the disks were dried the fluorescent signal on the disks was photographed under UV illumination and subsequently colonies on the disks were visualized by Coomassie blue staining (Raetz et al., 1982). Candidates for mutant clones were identified as colonies showing faint fluorescent spots and retrieved from the master dish maintained at 33°C with cloning cylinders. A mutant clone, UPS-1, was isolated in this manner and purified by limiting dilution. The phenotype of UPS-1 cells, described in this paper, has been stable for more than three months since the isolation of this clone.

Results

Effects of pH on C_6-NBD-PS Uptake

We first wanted to find conditions which inhibited endocytic uptake but not the non-endocytic uptake of fluorescent PS analogs. Since acidic treatment of cells had been shown to block clathrin-dependent endocytosis (Davoust et al., 1987; Sandvig et al., 1987; Heuser, 1989), we examined the effect of pH on C_6-NBD-PS uptake by CHO-K1 fibroblasts. CHO cell monolayers were incubated with C_6-NBD-PS/DOPC vesicles for 10 min at 37°C at various pH values, and subsequently back exchanged at 4°C to remove fluorescent lipid from the outer leaflet of the plasma membrane bilayer. The amount of intracellular fluorescent lipid was then determined by lipid extraction and analysis. Interestingly, C_6-NBD-PS uptake was enhanced at acidic pH (Fig. 1). The uptake approached a plateau value with decreasing pH, and at pH 6.0 was more than twice that at pH 7.4, the typical pH for cell culture (Fig. 1 *A*). More than 95% of the fluorescent lipid extracted from the cells following incubation at low pH was C_6-NBD-PS as determined by TLC analysis (data not shown), demonstrating that the low pH incubation conditions did not produce large amounts of other fluorescent lipids which might be preferentially incorporated by the cells. The uptake of C_6-NBD-PS was nearly linear during the first 10 min of incubation with 20 μM C_6-NBD-PS at either pH 6.0 or 7.4, and was directly proportional (at 10 min) to C_6-NBD-PS concentration, up to about 40 μM, at either pH (Fig. 1, *B* and *C*). Based on these data, we adopted standard incubation conditions in which 20 μM C_6-NBD-PS was incubated with cells for 10 min at 37°C. Non-specific permeabilization of the plasma membrane did not occur under these conditions since more than 95% of the cells exposed to pH 6.0 (or 7.4) excluded trypan blue.

Inhibition of C_6-NBD-PS Uptake

Translocation of various PS analogs across the plasma membrane bilayer has been studied in various cell types and is thought to be a protein-mediated process which is ATP-dependent and sensitive to sulfhydryl reagents (reviewed in

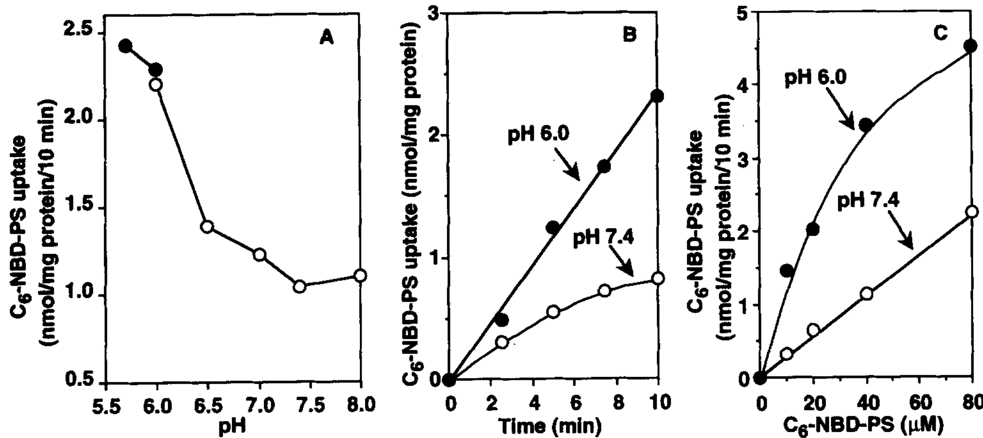


Figure 1. Effect of pH on C_6-NBD-PS uptake in CHO-K1 cells. (*A*) Cells were incubated with 20 μM C_6-NBD-PS for 10 min at 37°C in HBS (○) or ABS (●) at the indicated pH and the uptake quantified. (*B*) Time course for uptake of 20 μM C_6-NBD-PS in HBS (*pH 7.4*) or ABS (*pH 6.0*) at 37°C. (*C*) Concentration dependence of C_6-NBD-PS uptake. Cells were incubated in HBS (*pH 7.4*) or ABS (*pH 6.0*) with C_6-NBD-PS/DOPC (1:2, mol/mol) donor vesicles to give the indicated concentration of C_6-NBD-PS.

Schroit and Zwaal, 1991; Devaux, 1992; Zachowski, 1993). To learn whether C_6-NBD-PS uptake at acidic pH was also protein mediated, we studied the effects of various inhibitors on C_6-NBD-PS uptake. Cells were pretreated with deoxyglucose and NaN_3 in glucose-free medium at pH 6.0 or 7.4, and then incubated with C_6NBD-PS donor vesicles in the presence of the inhibitors. Under these conditions C_6-NBD-PS uptake was about 20% of that seen in control cells at either pH (Fig. 2). The ATP level in cells incubated in ABS (pH 6.0) was 93 ± 12% (n = 4) of that in cells incubated in HBS (pH 7.4), while incubation of cells in ABS^{+inhib} or HBS^{+inhib} for 10 min at 37°C reduced cellular ATP levels to about 10% of control values. NEM-treatment also clearly inhibited C_6-NBD-PS uptake (Fig. 2) although uptake in NEM-treated cells might be overestimated since fluorescence microscopy demonstrated that NEM treatment induced non-specific permeablization in about 5% of the cells. C_6-NBD-PS uptake was almost completely abolished at 4°C although transfer of the fluorescent lipid from donor vesicles to the plasma membrane readily occurred at 4°C (data not shown; see also Martin and Pagano, 1987). These results indicated that C_6-NBD-PS uptake at pH 6.0, as well as pH 7.4, was mediated by an energy-dependent and NEM-sensitive system(s).

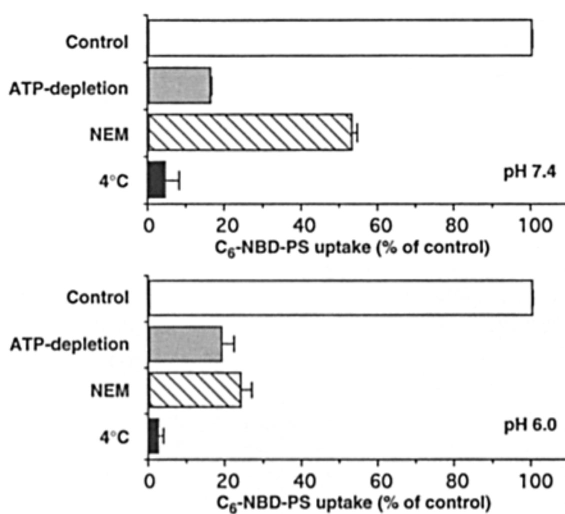


Figure 2. Inhibition of C_6-NBD-PS uptake. Untreated control cells, ATP-depleted, or NEM-treated cells (see Materials and Methods) were incubated with 20 μM C_6-NBD-PS in ABS (*pH 6.0*) or HBS (*pH 7.4*) for 10 min at 37°C, and the uptake quantified. Uptake at low temperature (10 min at 4°C) in untreated cells was also quantified following a 5 min preincubation at 4°C in ABS (*pH 6.0*) or HBS (*pH 7.4*). Values are expressed as a percentage of the uptake seen in untreated cells at either pH at 37°C, and are the means ±SD (n = 3).

Effects of pH on the Capacity of the Plasma Membrane to Accept C_6-NBD-PS and on the Loss of C_6-NBD-PS from Cells

We next determined whether the capacity of the outer leaflet of the plasma membrane bilayer to accept C_6-NBD-PS from donor vesicles was pH dependent, since alterations in the amount of C_6-NBD-PS which could partition into the plasma membrane might affect the amount of fluorescent lipid incorporated into intracellular membranes. To estimate the amount of C_6NBD-PS associated with the outer leaflet of the plasma membrane bilayer, cells were incubated with donor vesicles for 10 min at 37°C at either pH 7.4 or 6.0, and then the size of the "back-exchangeable" pool of C_6-NBD-PS was determined (see Materials and Methods). As shown

Table I. Effect of pH on the Amount of C_6-NBD-PS Associated with the Outer Leaflet of the Plasma Membrane Bilayer

pH*	Cell associated C_6-NBD-PS‡		Back-exchangeable C_6-NBD-PS§
	−Back-exchange	+Back-exchange	
		(nmol/mg protein)	
7.4	5.99 ± 0.79	1.73 ± 0.03	4.26
6.0	7.56 ± 0.49	3.64 ± 0.14	3.92

* CHO-K1 cells were incubated with 20 μM C_6-NBD-PS donor vesicles at the indicated pH for 10 min at 37°C.

‡ (*+Back-exchange*) After rinsing with DF-BSA, the cells were subjected to the back-exchange protocol (see Materials and Methods), washed with PBS, and the fluorescent lipids extracted, quantified, and normalized to cellular protein. (*−Back-exchange*) Values were obtained in the same manner, except the back-exchange procedure was omitted. Data represent the means ±SD (n = 3).

§ Determined by subtracting the amount of cell-associated C_6-NBD-PS obtained in the presence of back-exchange from that found in the absence of back-exchange.

in Table I, the amount of C_6-NBD-PS available for back-exchange was nearly the same at both pH values. In addition, we measured the spontaneous transfer of C_6-NBD-PS from donor to acceptor vesicles (Nichols and Pagano, 1982), and found that the half-time for equilibration between vesicle populations at pH 6.0 ($t_{1/2} \sim 35$ s) was nearly identical to that at pH 7.4 ($t_{1/2} \sim 37$ s).

We also examined the effect of pH on the loss of NBD–lipid fluorescence from cells, which might occur through degradation and/or secretion of the lipid. Cells were labeled with C_6-NBD-PS for 10 min at 37°C (pH 7.4), back-exchanged at 4°C, and then chased at 37°C in the presence of defatted BSA at either pH 6.0 or 7.4. The time course for the disappearance of the fluorescent lipid from the cells at pH 6.0 was nearly identical to that obtained at pH 7.4 (data not shown).

Simultaneous Labeling with C_6-NBD-PS and C_6-NBD-SM

We examined the possibility that the enhanced uptake of C_6-NBD-PS under acidic conditions might result from the induction of fusion or engulfment of donor vesicles by the cells. In these experiments, donor vesicles containing both C_6-NBD-PS and C_6-NBD-SM were used. Previous studies using CHO-K1 cells and C_6-NBD-SM at neutral pH showed that the lipid could be integrated into the outer leaflet of the plasma membrane bilayer at low temperature and subsequently be internalized into the cells via endocytosis at 37°C (Koval and Pagano, 1989). Furthermore, acidic treatment of cells is known to block clathrin-dependent endocytosis (Davoust et al., 1987; Sandvig et al., 1987; Heuser, 1989). Thus, if the pathway of C_6-NBD-SM uptake by the cells under acidic conditions was limited to endocytosis, acidic treatment would inhibit C_6-NBD-SM uptake. Conversely, if fusion or engulfment of donor vesicles by the cells caused the enhanced uptake of C_6-NBD-PS, the acidic treatment would induce enhanced uptake of C_6-NBD-SM as well as C_6-NBD-PS from the doubly labeled donor vesicles. As shown in Fig. 3, acidic treatment inhibited C_6-NBD-SM uptake to 40% of control values at pH 7.4, while the uptake of C_6-NBD-PS was enhanced, as shown in Fig. 1 *A*. These results demonstrate that acid-induced fusion or engulfment of the donor vesicles by the cells was negligible under the experimental conditions used in this study. The enhanced C_6-NBD-PS uptake at acidic pH was also not due to a secondary effect on the inhibition of endocytosis since a hypertonic treatment which blocks clathrin-dependent endocytosis (Daukas and Zigmond, 1985; Heuser and Anderson, 1989) also reduced C_6-NBD-SM uptake, but did not affect C_6-NBD-PS uptake (Fig. 3).

Intracellular Distribution of C_6-NBD-PS

The effect of pH on the intracellular distribution of C_6-NBD-PS was examined by fluorescence microscopy. When the cells were incubated at 37°C and pH 7.4 with C_6-NBD-PS/DOPC vesicles various intracellular organelles including the endoplasmic reticulum and the nuclear envelope were labeled (data not shown; refer to Fig. 7 *A*). A similar pattern of intracellular fluorescence was seen when cells were incubated at pH 6.0, except that the specimen was more intensely labeled (data not shown). The intracellular distribution of C_6-NBD-PS can best be explained by a mechanism in which this fluorescent lipid is internalized by transbilayer movement at the plasma membrane rather than by endocytosis (Sleight and Pagano, 1985; Martin and Pagano, 1987; Kobayashi and Arakawa, 1991). Once present at the cytosolic leaflet of the plasma membrane, C_6-NBD-PS can transfer into other intracellular membranes since it exhibits rapid spontaneous transfer between membranes in vitro (Tanaka and Schroit, 1986).

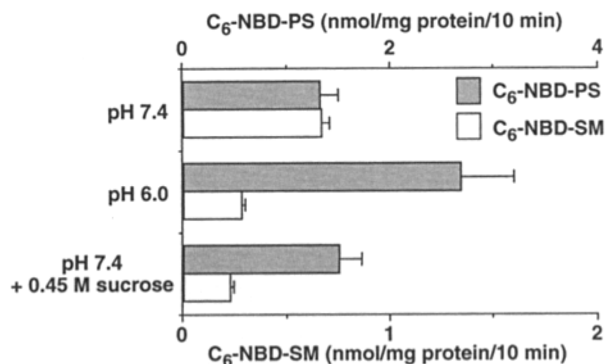


Figure 3. Cellular uptake of fluorescent lipids from donor vesicles containing both C_6-NBD-PS and C_6-NBD-SM. CHO-K1 cells were preincubated in the indicated media (HBS, ABS, or HBS + 0.45 M sucrose) for 5 min at 37°C, incubated for 10 min 37°C with 80 μM donor vesicles comprised of C_6-NBD-PS/C_6-NBD-SM/DOPC (1:1:2, mol/mol/mol) in the indicated media, and then subjected to back exchange at 4°C. The cellular lipids were then extracted, separated by TLC, and the amount of C_6-NBD-PS and C_6-NBD-SM quantified (see Materials and Methods). Data are the means ±SD (n = 3).

Effects of Cytosolic Acidification on C_6-NBD-PS Uptake

Incubation of cells with acidic medium is known to cause acidification of the cytosol (L'Allemain et al., 1984; Davoust et al., 1987). Indeed, we observed that the cytosolic pH approached 6.0 after incubation of the CHO-K1 cells in ABS (pH 6.0). To learn if C_6-NBD-PS uptake could be enhanced by intracellular acidification in the absence of extracellular acidification, we next used a "NH_3 load and release" protocol (Boron 1983). When CHO-K1 cells preincubated in HBS (pH 7.4) containing 30 mM NH_4Cl were transferred to NH_3-free HBS (pH 7.4), the cytosol was rapidly acidified to about pH 6.6, however, this value returned to almost the original pH within 3 min (Fig. 4 *B*). When the NH_3-loaded cells were transferred to NH_3-free medium containing 1 mM amiloride, an inhibitor of the Na^+/H^+ exchanger which is the main machinery for pH homeostasis (Grinstein et al., 1989), the cytosol was acidified to about pH 6.3, and the acidification remained stable for at least 10 min (Fig. 4 *C*). As expected, when the NH_3-loaded cells were transferred to 30 mM NH_4Cl-containing HBS (pH 7.4) to eliminate any NH_3 gradient, there was no significant acidification of the cytosol even in the presence of amiloride (Fig. 4 *D*).

As shown in Fig. 5, when the cells were incubated with C_6-NBD-PS under conditions which induced a transient acidification of the cytosol, C_6-NBD-PS uptake increased by about 20%, compared with the control value determined

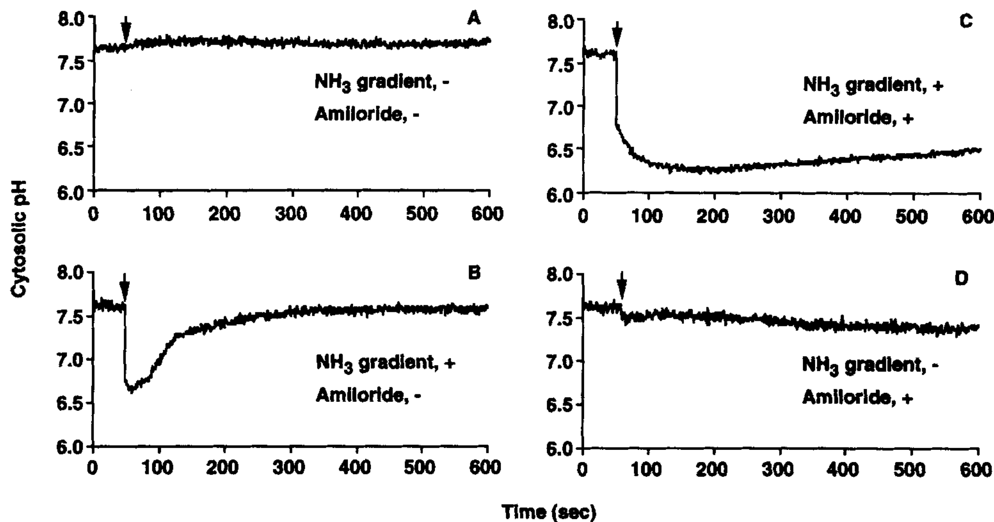


Figure 4. Cytosolic acidification by NH_3 load and release. CHO-K1 cell monolayers loaded with BCECF were preincubated in HBS (pH 7.4) containing 30 mM NH_4Cl for 5 min at 37°C and the cytosolic pH was monitored as described (see Materials and Methods). At the times indicated by the arrows the monolayers were transferred to the following buffers. (*A*) HBS (pH 7.4) containing 30 mM NH_4Cl; (*B*) HBS (pH 7.4); (*C*) HBS (pH 7.4) containing 1 mM amiloride; (*D*) HBS (pH 7.4) containing 30 mM NH_4Cl and 1 mM amiloride.

without the NH_3-gradient or amiloride. Under the conditions inducing a stable acidification of the cytosol, C_6-NBD-PS uptake was further enhanced by about 50%. This enhancement was not due to "secondary" effects of amiloride, since addition of amiloride in the absence of a NH_3-gradient did not affect C_6-NBD-PS uptake (Fig. 5). These results demonstrate that the C_6-NBD-PS uptake was enhanced by acidification of the cytosol even when this occurred in the absence of acidification of the extracellular medium. These results also eliminate the possibility of a simple equilibrium mechanism for C_6-NBD-PS uptake at acidic pH in which protonated PS molecules more easily cross the plasma membrane and are subsequently trapped there once de-protonation occurs. Such a mechanism is very unlikely since acidification of the cytosol in the absence of acidification of the extracellular medium should result in an unfavorable transmembrane pH gradient to trap acidic molecules like C_6-NBD-PS in the interior of the cell (Eastman et al., 1991).

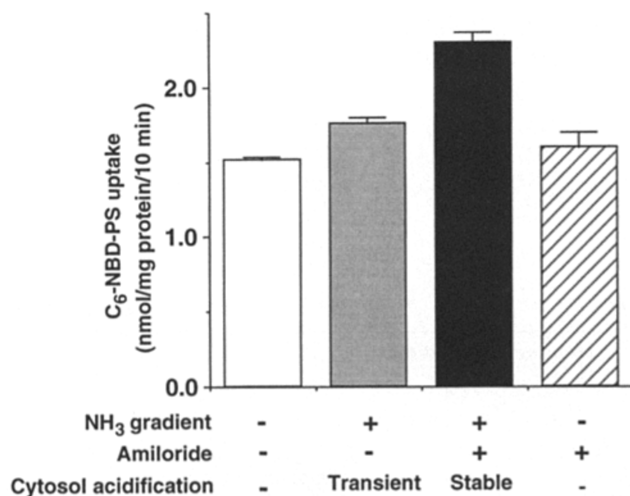


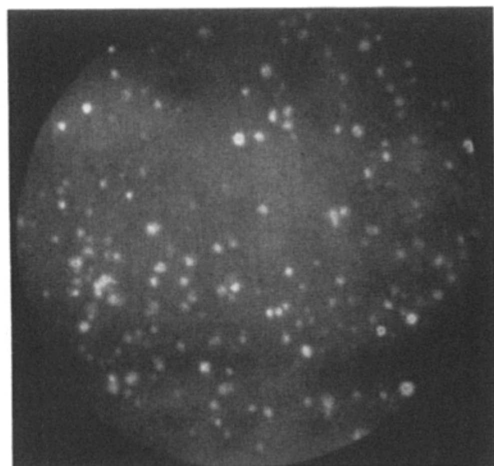
Figure 5. Effects of cytosolic acidification on C_6-NBD-PS uptake. CHO-K1 monolayers were preincubated in HBS (pH 7.4) containing 30 mM NH_4Cl for 5 min at 37°C. The cells were then rinsed with 2 ml of HBS (pH 7.4) in the presence (+) or the absence (−) of an NH_3 gradient and/or 1 mM amiloride, and further incubated for 10 min at 37°C in 1 ml of HBS (pH 7.4) containing 20 μM C_6-NBD-PS donor vesicles in the presence (+) or the absence (−) of an NH_3 gradient and/or 1 mM amiloride. The nature of the cytosolic acidification, determined from the results in Fig. 4, and the amount of cell-associated C_6-NBD-PS determined after back-exchange are shown. Data are the means ±SD (n = 3).

Selection of Mutant Cells Defective in Non-endocytic Uptake of Fluorescent PS Analogs under Acidic Conditions

Taking advantage of our findings that acidic conditions inhibited the endocytic membrane flow and enhanced C_6-NBD-PS uptake by a non-endocytic pathway in CHO-K1 cells, we developed a screening system to isolate mutant cells defective in the uptake of fluorescent PS analogs by the non-endocytic pathway. After formation of replica colonies of CHO-K1 cells on polyester disks, the replica disks were incubated with fluorescent PS analogs under acidic conditions. As shown in Fig. 6, fluorescence was associated with colonies visualized by Coomassie staining whereas there was no appreciable fluorescent signal when the incubation was carried out at 4°C (data not shown) indicating that these procedures were useful as an in situ assay of fluorescent PS analog uptake. 1-C_6-2-C_{12}-NBD-PS was routinely used as the standard probe in the screening since we found that 1-C_6-2-C_{12}-NBD-PS produced a stronger signal than other fluorescent PS analogs including C_6-NBD-PS.

Isolation of UPS-1, a CHO Mutant Cell Line Defective in Uptake of Fluorescent PS Analogs

After screening about 20,000 colonies of mutagenized CHO-K1 cells, we obtained one clone partially defective in uptake of 1-C_6-2-C_{12}-NBD-PS and named it UPS-1 (*u*ptake of fluorescent *PS* analogs). Uptake activity of 1-C_6-2-C_{12}-NBD-PS in UPS-1 cells at pH 6.0 was only 30% of that of the wild type (Table II). To compare energy-dependent uptake, we also determined the "ATP-dependent uptake activity" by subtracting the activity under ATP-depletion conditions from that under standard conditions. The ATP-dependent uptake of 1-C_6-2-C_{12}-NBD-PS in UPS-1 cells was less than



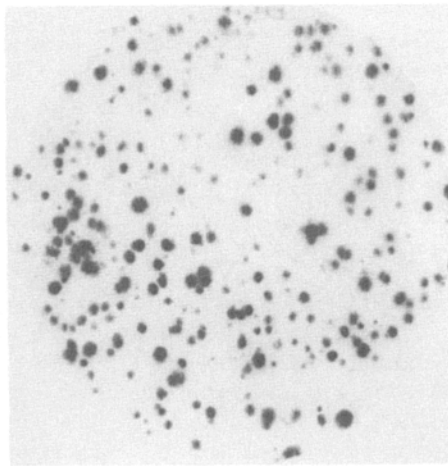


Figure 6. In situ assay for uptake of a fluorescent PS analog with replica colonies. A polyester disk forming replica colonies of the wild type CHO cells was incubated with 20 μM 1-C_6-2-C_{12}-NBD-PS at pH 6.0 at 37°C for 1 h. After quenching extracellular fluorescent lipids with $Na_2S_2O_4$, fluorescence on the disk was photographed under UV illumination (*left*), and subsequently colonies on the disks were visualized by Coomassie blue staining (*right*).

25% of the wild type level (Table II). In addition, when C_6-NBD-PS and 1-oleoyl-C_6-NBD-PS were used as probes, UPS-1 cells showed similar deficiencies in ATP-dependent uptake of these fluorescent PS analogs, while there was no difference in uptake of 1-palmitoyl-2-C_6-NBD-*sn*-glycero-3-phosphocholine between UPS-1 and the wild type cells. Although the PS transbilayer transport system is thought to also transport phosphatidylethanolamine (reviewed in Schroit and Zwaal, 1991; Devaux 1992; Zachowski, 1993), a defect in uptake of 1-palmitoyl-2-C_6-NBD-*sn*-glycero-3-phosphoethanolamine in UPS-1 cells was not obvious (Table II) apparently because this probe was not efficiently recognized by the PS transbilayer transport system (Colleau et al., 1991; Connor et al., 1992). When CHO-K1 cells were labeled with 1-palmitoyl-2-C_6-NBD-*sn*-glycero-3-phosphoethanolamine or 1-palmitoyl-2-C_6-NBD-*sn*-glycero-3-phosphocholine at pH 7.4 at 37°C for 10 min, a punctate pattern of intracellular fluorescence was observed by fluorescence microscopy which was inhibited under acidic conditions (data not shown), suggesting that these fluorescent phosphatidylethanolamine and phosphatidylcholine analogs were internalized in CHO-K1 cells mainly by the endocytic pathway at pH 7.4 at 37°C. Moreover we found that 1-hexanoyl-2-C_6-NBD-PS was not appreciably incorporated by the wild type or UPS-1 cells, supporting the idea that the acyl chain composition as well as the nature of polar head group are important in determining the transbilayer movement of PS. These results suggest that a non-endocytic pathway for fluorescent PS analogs was specifically impaired in UPS-1 cells. It should also be noted that there was no difference in temperature sensitivity of either uptake activity of fluorescent PS analogs or cell growth between UPS-1 cells and the wild type cells when they were examined at 33°, 37°, and 39°C (data not shown).

A Specific Defect in a Non-endocytic Pathway Was Responsible for Uptake of Fluorescent PS Analogs in UPS-1 Cells

To confirm that the deficiency in UPS-1 cells was restricted to the non-endocytic pathway for uptake of fluorescent PS

Table II. Uptake of Various Fluorescent Analogs of Phospholipids in CHO-K1 and UPS-1 Cells under Acidic Conditions

NBD-lipids	Cells	NBD-lipid uptake activity*		
		ABS	ABS^{+inhib}	ATP-dependent
		(nmol/mg protein/10 min)		
1-C_6-2-C_{12}-NBD-PS	CHO-K1	4.00 ± 0.26	0.56 ± 0.07	3.44
	UPS-1	1.27 ± 0.33	0.48 ± 0.05	0.79
1-Palmitoyl-2-C_6-NBD-PS	CHO-K1	2.95 ± 0.40	0.29 ± 0.10	2.66
	UPS-1	1.19 ± 0.16	0.40 ± 0.10	0.79
1-Oleoyl-2-C_6-NBD-PS	CHO-K1	1.28 ± 0.10	0.29 ± 0.10	0.99
	UPS-1	0.51 ± 0.03	0.26 ± 0.05	0.25
1-Hexanoyl-2-C_6-NBD-PS	CHO-K1	<0.02	not tested	–
	UPS-1	<0.02	not tested	–
1-Palmitoyl-2-C_6-NBD-PC‡	CHO-K1	0.36 ± 0.01	0.10 ± 0.03	0.24
	UPS-1	0.35 ± 0.04	0.10 ± 0.03	0.24
1-Palmitoyl-2-C_6-NBD-PE§	CHO-K1	0.78 ± 0.26	0.11 ± 0.05	0.67
	UPS-1	0.67 ± 0.20	0.10 ± 0.05	0.57

* Monolayers of CHO-K1 and UPS-1 cells were incubated with 20 μM NBD-lipid donor vesicles at 37°C for 10 min in ABS (pH 6.0) for standard conditions or in ABS^{+inhib} (pH 6.0) for ATP-depletion conditions. After back-exchange, NBD-lipid fluorescence in the cells was quantified, and is shown as the mean ± SD (n = 3). ATP-dependent uptake activity was estimated by subtracting the uptake activity under ATP-depletion conditions from that under standard conditions.
‡ 1-palmitoyl-2-C_6-NBD-*sn*-glycero-3-phosphocholine.
§ 1-palmitoyl-2-C_6-NBD-*sn*-glycero-3-phosphoethanolamine.

analogs, cells were labeled with three different types of NBD-phospholipid analogs at pH 7.4 and observed under the fluorescence microscope. When the cells were incubated with 1-C_6-2-C_{12}-NBD-PS, various intracellular organelles of the wild type cells were strongly labeled (Fig. 7 *A*) while the intracellular fluorescece of the UPS-1 cells was much fainter (Fig. 7 *B*), consistent with the results of the uptake assay at pH 6.0 (Table II). In contrast, when the cells were incubated with C_6-NBD-SM to monitor endocytic membrane flow, UPS-1 cells showed no significant difference in the distribution of punctate, intracellular fluorescence, compared to the wild type cells (Fig. 7, *C* and *D*). This observation indicated that lipid internalization by the endocytic pathway in UPS-1 cells was normal. We also used 1-palmitoyl-2-C_6-NBD-*sn*-glycero-3-phosphate, a fluorescent phosphatidic acid analog to make a qualitative comparison of other intracellular membranes in UPS-1 and the wild type cells. This lipid is dephosphorylated to fluorescent diacylglycerol at the cell surface, and after spontaneous transbilayer movement at the plasma membrane, labels various intracellular membranes such as the nuclear envelope and endoplasmic reticulum (Pagano and Longmuir, 1985). Incubation of cells with the fluorescent phosphatic acid analog showed similar labeling patterns in both cell types (Fig. 7, *E* and *F*), indicating that there were no obvious differences in the amount or distribution of these intracellular membranes in the two cell types.

The UPS-1 Phenotype Was Unrelated to Multidrug Resistance

Overexpression of P-glycoprotein (*mdrl* gene product) is known to give the host cells a multidrug-resistant phenotype, which results from accelerated efflux of various amphipathic drugs from the cells (reviewed in Gottesman and Pastan, 1993). To test whether the phenotype of UPS-1 cells was related to multidrug resistance, intracellular labeling with 1-C_6-2-C_{12}-NBD-PS at physiological pH was compared among the wild type CHO-K1, UPS-1, and ALLN^{r50} cells, a P-glycoprotein-overproducing CHO variant (Sharma et al., 1992). As shown in Fig. 8, the fluorescence intensity of labeled ALLN^{r50} cells was similar to that of the wild type CHO-K1 cells while intracellular labeling of UPS-1 cells was much fainter. Moreover, exposure of these cells to 0.1 mM verapamil, which competitively suppresses drug efflux via the P-glycoprotein (Yusa and Tsuruo, 1989), did not affect the labeling of these cells with 1-C_6-2-C_{12}-NBD-PS (Fig. 8). We further confirmed that the wild type CHO-K1 and UPS-1 cells showed similar sensitivity to colchicine, while ALLN^{r50} cells were much more resistant to colchicine (data not shown). These results indicated that the uptake deficiency of fluorescent PS analogs by UPS-1 cells was unrelated to the multidrug resistance conferred by overproduction of P-glycoprotein.

Discussion

Cytosolic Acidification Stimulates Uptake of C_6-NBD-PS by a Non-endocytic Pathway

In the present study, we showed that acidic treatment of CHO-K1 cells enhanced the uptake of C_6-NBD-PS without inducing non-specific permeabilization of the cells or altering the rate of disappearance of C_6-NBD-PS from cells. The major pathway for C_6-NBD-PS uptake in CHO-K1 cells at 37°C under acidic (as well as neutral) conditions was most likely through the ATP-dependent PS transporter. This conclusion is supported by our observations of: (*a*) the intracellular distribution of C_6-NBD-PS fluorescence; (*b*) the inhibition of C_6-NBD-PS uptake by ATP depletion and NEM treatment; and (*c*) the differential uptake of C_6-NBD-PS and C_6-NBD-SM from doubly labeled lipid vesicles. Furthermore, we demonstrated that cytosolic acidification, without extracellular acidification, also enhanced C_6-NBD-PS uptake. These results suggest that cytosolic acidification stimulates the PS translocase system at the plasma membrane of CHO-K1 cells.

The physiological significance of the stimulation of the PS translocase at acidic pH is not known. It has been suggested that this transporter may play a crucial role in maintaining an asymmetric distribution of PS at the plasma membrane (reviewed in Schroit and Zwaal, 1991; Devaux, 1992; Zachowski, 1993), and it is likely that plasma membrane lipid asymmetry may be perturbed during vesicle fusion and fission events which occur as normal consequences of endocytosis, secretion, and cell division. Various organelles including endosomes, secretory vesicles, and the *trans*-Golgi network are mildly acidic (pH 5.5–6.5) (reviewed in Anderson and Orci, 1988; Forgac, 1989). The perturbation of lipid asymmetry at the plasma membrane during secretion and endosome recycling events might be accompanied by leakage of protons from these acidic compartments into the cytosol, causing a "local acidification" which enhances PS translocase activity. It is unknown whether low cytosolic pH directly activates the PS translocase or indirectly affects other factor(s) which can stimulate its activity. If there is an interaction between the PS transporter and membrane cytoskeleton, such interaction might participate in regulation of the PS transport activity since cytosolic acidification is known to cause redistribution of fodrin in MDCK cells (Eskelinen et al., 1992).

A Mutant Cell Line Partially Defective in the Non-endocytic Uptake of Fluorescent PS Analogs

Although genetic approaches can be very useful for biological studies, no successful report of the isolation of mutant cells defective in the uptake of PS or its analogs has been reported. Furthermore, if one wants to select mutant cells defective in the non-endocytic pathway of PS uptake, it is important to use a screening method which minimizes unwanted background due to endocytosis of the PS probe. Although energy depletion or chemical modification of cells and low temperature conditions are known to block endocytosis, these treatments are difficult to employ in mutant screening since they also inhibit the non-endocytic uptake pathway (Fig. 2). Anucleated cells such as human erythrocytes, in which endocytosis does not occur, are advantageous to investigate the transbilayer movement of PS in the absence of endocytosis, however, these cells cannot serve as the parental cells for mutant selection. Our finding that acidic conditions inhibited internalization of membrane lipids by the endocytic pathway and enhanced uptake of C_6-NBD-PS by the non-endocytic pathway in CHO-K1 cells allowed us to develop a convenient screening method for mutants in the

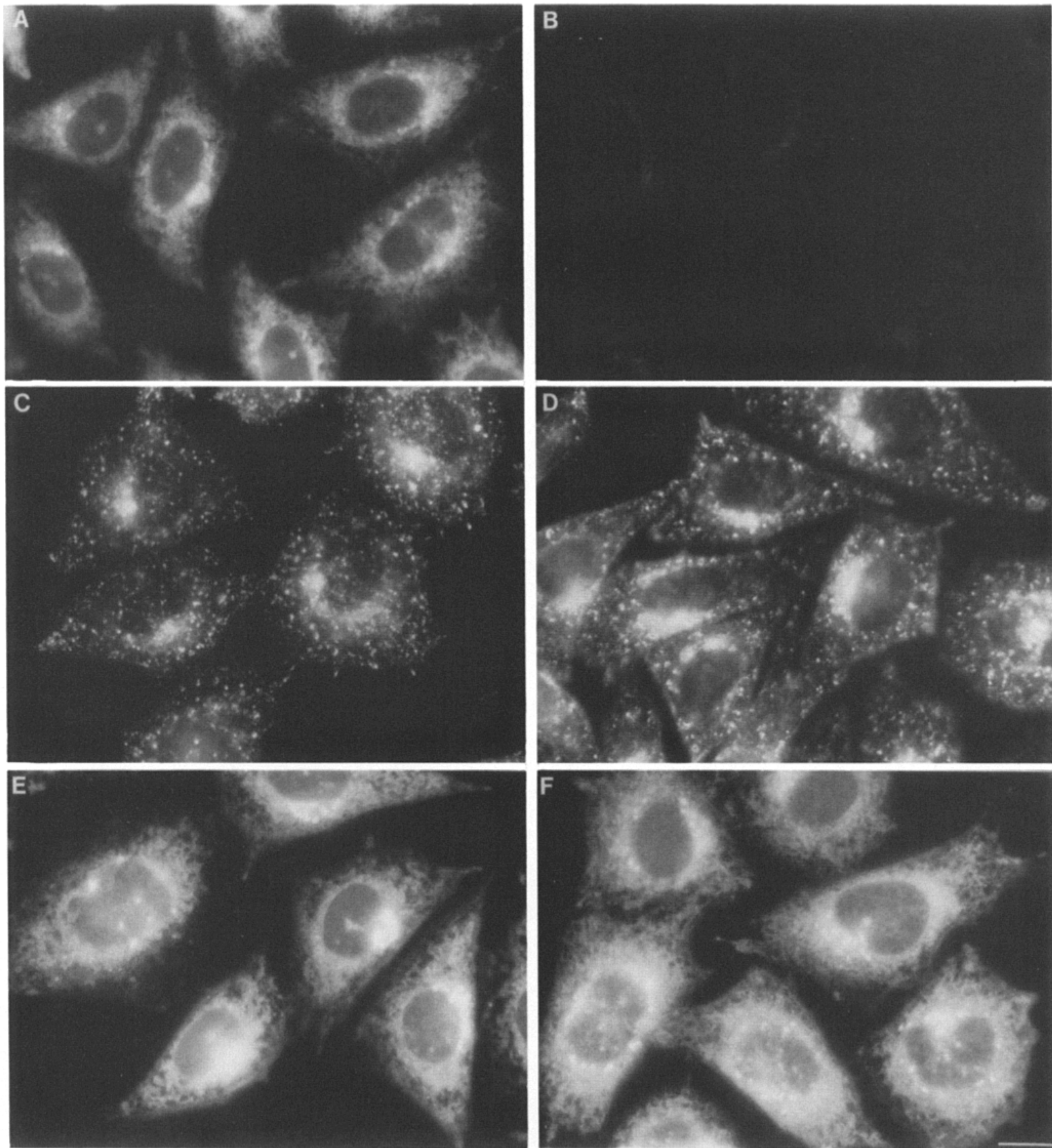
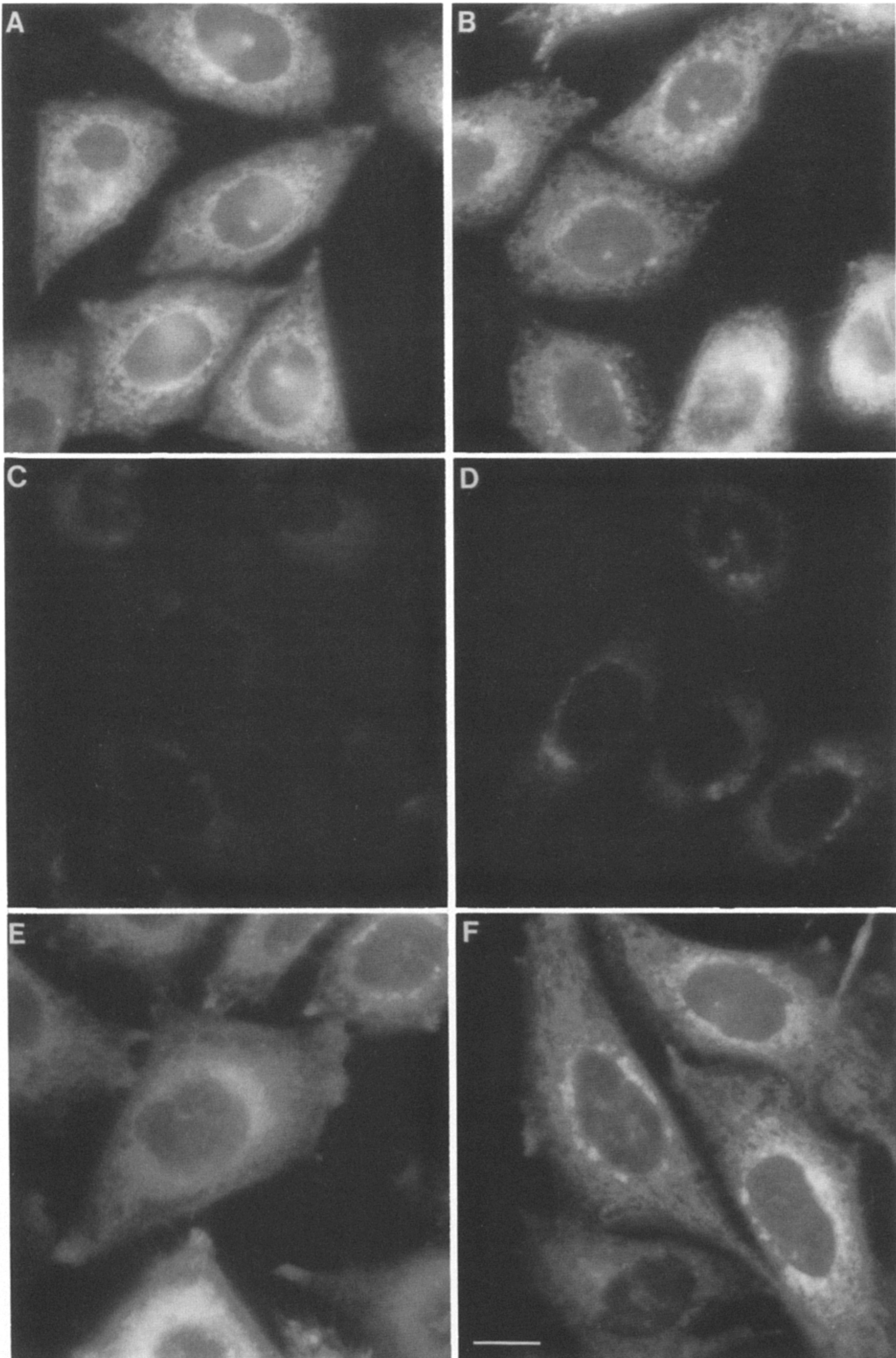


Figure 7. Fluorescence microscopy of CHO-K1 and UPS-1 cells labeled with various NBD-phospholipids. CHO-K1 (*A*, *C*, and *E*) and UPS-1 (*B*, *D*, and *F*) cells were incubated with NBD-phospholipid donor vesicles in HBS (pH 7.4) for 10 min at 37°C, back-exchanged at 4°C, and observed under the fluorescence microscope. (*A* and *B*) 20 μM 1-C_6-2-C_{12}-NBD-PS; (*C* and *D*) 20 μM C_6-NBD-SM; (*E* and *F*) 10 μM 1-palmitoyl-2-C_6-NBD-*sn*-glycero-3-phosphate. Each pair of photomicrographs (*A* and *B*) (*C* and *D*), and (*E* and *F*) was exposed and printed identically. Bar, 10 μm.

non-endocytic pathway and led us to obtain one mutant clone, UPS-1, which is defective in the uptake of fluorescent PS analog by the non-endocytic pathway.

Several lines of evidence suggested that a non-endocytic pathway responsible for uptake of fluorescent analogs was specifically impaired in UPS-1 cells. First, substrate specificity of NBD-phospholipid uptake revealed that UPS-1 cells showed a deficiency in uptake of NBD-PS analogs which are known to be good probes for transbilayer PS movement, but no deficiency in uptake of other NBD-phospholipids (Table II and Fig. 7). Second, fluorescence microscopy demonstrated that UPS-1 cells internalized C_6-


A
B
C
D
E
F

NBD-SM like the wild type cells (Fig. 7, *C* and *D*) indicating that UPS-1 cells sustained normal membrane flow by endocytosis. It is therefore unlikely that non-specific membrane perturbations resulted in the UPS-1 phenotype. Last, since UPS-1 cells showed an uptake deficiency of fluorescent PS analogs at both acidic and neutral pH (Table II and Fig. 7), the UPS-1 phenotype was not due to an impairment of acidic stimulation of PS uptake. While uptake of NBD-PS was substantially impaired in UPS-1 cells, some ATP-dependent uptake of fluorescent PS analogs by UPS-1 cells relative to the wild type cells was still observed (Table II). These observations might imply that there were at least two different types of PS transport systems in the wild type cells and only one of these putative isotypes was impaired in UPS-1 cells. Alternatively, only a partial inactivation of the responsible protein may have occurred in UPS-1 cells. It is also possible that one gene of a functional diploid of the same PS transport system might be disrupted in the UPS-1 cells. However, this possibility seems unlikely since ATP-dependent uptake activity of fluorescent PS analogs in UPS-1 was much less than half of the wild type levels (Table II). Since the net uptake of fluorescent PS represents a balance between the influx and efflux of the fluorescent lipid, another possible explanation for the UPS-1 phenotype is that it results from an enhanced efflux of fluorescent PS from the cells. We could not completely eliminate this possibility because no specific inhibitors of PS influx via transbilayer movement are currently available. However, we were able to demonstrate that mdr-1 overproduction did not confer the UPS-1 phenotype (Fig. 8).

Overproduction of mdrl P-glycoprotein confers resistance to various chemotherapeutic agents since the P-glycoprotein can function as an ATP-driven transmembrane efflux pump of these drugs (reviewed in Gottesman and Pastan, 1993). Based on observations that the P-glycoprotein recognizes various types of compounds without significant structural similarities but with a common amphipathic property, it has been hypothesized that the P-glycoprotein might be a transbilayer lipid flippase with no strict substrate specificity (Higgins and Gottesman, 1992). Although the phenotype of UPS-1 cells was clearly unrelated to multidrug resistance conferred by overproduction of mdrl P-glycoprotein (Fig. 8), the machinery for PS transbilayer movement could be a member of "ATP-binding cassette transporters (Higgins, 1992)." Indeed, the hypothesis of lipid transbilayer movement by ATP-binding cassette transporters was recently substantiated. The mouse *mdr2* gene had been initially isolated as a gene highly homologous to *mdrl* gene, however, overproduction of the mdr2 protein in cells did not cause any appreciable drug resistance (Gros et al., 1988). A recent study with homozygous mutant mice demonstrated that disruption of the *mdr2* gene resulted in dysfunction of excretion of phosphatidylcholine from the apical membrane (bile canalicular membrane) of hepatocytes into bile ducts and strongly suggested that the mdr2 protein functioned as an outward transporter of phosphatidylcholine at the apical membrane in hepatocytes (Smit et al., 1993). On the other hand, an inward transbilayer movement of phosphatidylcholine at the plasma membrane has also been suggested to occur in some cultured mammalian cells as seen with fluorescent analogs of phosphatidylcholine (Sleight and Abanto, 1989), and a similar uptake system for phosphatidylcholine at the plasma membrane in yeast cells was recently demonstrated (Kean et al., 1993). Hopefully, mutant cells like UPS-1 will be useful not only for investigation of the physiological meaning of these lipid transport systems but also for cloning the genes responsible for them by functional rescue methodology.

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Figure 8. Intracellular labeling of CHO-K1, UPS-1, and multidrug-resistant cells with a fluorescent PS analog in the presence or absence of verapamil. After incubation in HBS (pH 7.4) in the presence (*B*, *D*, and *F*) or absence (*A*, *C*, and *E*) of 0.1 mM verapamil for 5 min at 37°C, CHO-K1 (*A* and *B*), UPS-1 (*C* and *D*) and ALLN^{r50} (*E* and *F*) cells were incubated with 20 μM 1-C_6-2-C_{12}-NBD-PS donor vesicles in HBS (pH 7.4) in the presence or absence of 0.1 mM verapamil for 10 min at 37°C, back-exchanged at 4°C, and observed under the fluorescence microscope. All photomicrographs were exposed and printed identically. Bar, 10 μm.

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