Cytometry 28:36–41 (1997)

Verapamil Inhibition of Enzymatic Product Efflux Leads to Improved Detection of β-Galactosidase Activity in *lacZ*-Transfected Cells

Martin Poot* **and Seksiri Arttamangkul**
Department of Biosciences, Molecular Probes, Inc., Eugene, Oregon

Received 5 September 1996; Accepted 27 November 1996

The β-galactosidase activity encoded by the *lacZ* gene of *Escherichia coli* is widely used to monitor successful expression of transfected genes. Fluorogenic substrates allow detection of enzyme activity in viable cells, which, subsequently, can be selected for further study on the basis of fluorescence emission. We analyzed three fluorogenic substrates (FDG, C_{12}FDG, and CMFDG), all of which incorporate fluorescein as their fluorophore, regarding intensity of fluorescent signal and selectivity toward the transfected β-galactosidase activity vs. lysosomal enzyme activity. Among these substrates, 5-chloromethylfluoresecein di-β-D-galactopyranoside (CMFDG) showed the strongest selectivity toward the *lacZ*-encoded enzyme activity. An attempt to improve this selectivity by alkalinization of the lysosomal pH with chloroquine, such that the endogenous enzyme would be exposed to a suboptimal pH, led to significant cell death. In contrast, inhibition of dye efflux with verapamil enhanced the selectivity of CMFDG toward the *lacZ*-encoded enzyme activity by approximately threefold. Incubation with probenecid, on the other hand, showed little effect. Cytometry 26:36–41, 1997. © 1997 Wiley-Liss, Inc.

Key terms: β-galactosidase; fluorochromasia; *lacZ*, lysosomal enzyme activity; verapamil; FDG; CMFDG; fluorescence

Identification and selection of those cells that have taken up and express exogenously added DNA are pivotal to studies involving gene transfer. In this type of experiment, the gene of interest is embodied within a plasmid construct that contains a reporter gene. Reporter genes encode detectable markers, such as a surface antigen (10,12), an enzyme activity (17), or the green fluorescent protein (19). Modulation of the pattern of cell surface antigens may alter the behavior of a cell in a multicellular environment. Another weakness applying both to the use of surface antigens and to the green fluorescent protein is that the sensitivity of reporter detection is determined by the number of antigens incorporated into the plasma membrane or the number of green fluorescent protein molecules synthesized by the cell. In contrast, by using an enzyme activity as a reporter of gene expression, the signal can be amplified by the action of the enzyme upon a suitable substrate. Thus, depending on the intrinsic strength of the signal generated by the product of enzyme action and the contribution of endogenous enzyme activity of the cell, a signal of desired strength can be obtained.

The most widely used reporter enzyme, β-galactosidase, is encoded by the *lacZ* gene of *Escherichia coli* (16). Its activity leads to the formation of visible precipitates resulting from enzymatic cleavage of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) or to red fluorescence emission if it is monitored with resorufin β-D-galactopyranoside (18). Because these substrates are not plasma-membrane permeant, the cells under study have to be fixed or permeabilized before assay (18). This requirement and the resultant death of the cells preclude the use of these substrates in studies in which cells have to be recultured or injected into blastocysts. Hence, substrates that permeate into intact cells have been developed. Synthesis of mono- or digalactopyranosides of fluorescein leads to dyes with reduced fluorescence. Dye fluorescence is recovered after the sugar moieties are removed by enzyme action (17). This characteristic, termed fluorochromasia, allows detection of very low levels of substrate turnover. Because the bis-conjugated fluorescein (fluorescein di-β-D-galactopyranoside; FDG) is essentially nonfluorescent and the monoadduct (fluorescein mono-β-D-galactopyranoside) exhibits a fluorescence level of 8% of fluorescein (5), FDG will afford the highest sensitivity for detection of enzymatic turnover.

In intact cells, FDG showed limited sensitivity toward β-galactosidase action, which has been thought to result from a low rate of dye uptake. To overcome this problem,

*Correspondence to: Martin Poot, PhD, Department of Biosciences, Molecular Probes, Inc., P.O. Box 22010, Eugene, OR 97402.

a hypotonic shock protocol has been developed (11). In another attempt to enhance the uptake of fluorescein-based enzyme substrates, FDG was rendered more lipophilic by adding an alkyl chain to the fluorescein moiety (20). Apart from limited substrate uptake, leakage of products of enzymatic hydrolysis from the cell may occur. Leakage of the product of enzyme action (fluorescein) has previously been invoked to explain some problems encountered with FDG in experiments aiming at β-galactosidase detection in intact cells (7). The addition of a thiol-reactive chloromethyl group to fluorescein diacetate was shown to lead to labeling of intracellular thiols (13). Following this observation, it was reasoned that modification of the FDG dye with a chloromethyl group would enhance the retention of fluorescent product inside a cell. To test this hypothesis, we used a cell line that stably expresses *lacZ*-encoded β-galactosidase activity under the control of a murine leukemia virus promoter (CB2) and the nontransfected parental cell line (NIH 3T3). Comparison of this pair of cell lines allows ascertainment of the contribution of endogenous, lysosomal vs. transfected, *lacZ*-encoded enzyme activity. We analyzed several strategies to improve the selectivity of FDG, 5-dodecanoylaminofluorescein di-β-D-galactopyranoside (C_{12}FDG), and 5-chloromethylfluorescein di-β-D-galactopyranoside (CMFDG) for the *lacZ*-encoded enzyme activity.

MATERIALS AND METHODS

Chemicals

Chloroquine, propidium iodide, and the enzyme substrates FDG, C_{12}FDG, and CMFDG were from Molecular Probes, Inc. (Eugene, OR). Cell culture media, fetal bovine serum (FBS), and sterile tissue culture supplies were obtained from GIBCO-BRL (Gaithersburg, MD). All other reagents were from Sigma Chemical Co. (St. Louis, MO) and were of the highest purity available.

Cells and Culture

Mouse NIH 3T3, CRE BAG 2 (CB2) (15) fibroblasts were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in Dulbecco's modification of Eagle's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 μg gentamicin, 300 μg L-glutamine per ml of culture medium, and 10 mM HEPES, pH 7.4, in a humidified atmosphere of 5% CO_2 in air. Cells were subcultured every 2-3 days by trypsinization using 0.05% trypsin and 0.02% EDTA in phosphate-buffered saline (PBS). For experimentation, cells were trypsinized, collected by centrifugation, and resuspended at a density of 10^6 cells per ml in prewarmed culture medium supplemented with 10% FBS. Cell suspensions were incubated at 37°C in cell culture medium with fluorogenic substrates at concentrations and time intervals as indicated for each of the experiments. Cell viability was determined by adding 5 μM of propidium iodide shortly before assay by flow cytometry.

Flow Cytometry

After incubation with enzyme substrates, cell suspensions were analyzed by flow cytometry using a FACS-Vantage instrument (Becton and Dickinson Immunocytometry Systems, San Jose, CA) equipped with an argon laser (Coherent Enterprise laser, Coherent, Inc., Santa Clara, CA) set at a light-stabilized output of 100 mW of the 488 nm beam. Fluorescence emission was collected with interference band-pass filter sets for the spectral region of 530 ± 15 nm (''green'') and 630 ± 11 nm (''red''). The sheath fluid was made of 0.9% NaCl, and typical sample flow rates were between 200 and 400 particles per second. Data were stored as list-mode files onto the hard disk of a Macintosh Quadra 650 microcomputer and were analyzed with software supplied by the manufacturer. Viable cells were selected electronically by excluding signals from cells staining positive with propidium iodide.

High-Pressure Liquid Chromatography Analysis of Effluxed Products

Aliquots of 10×10^6 CB2 cells were incubated with 50 μM of CMFDG for 30 min in 1 ml of cell culture medium in the presence or absence of 200 μM verapamil. Cells were collected by centrifugation, and the supernatant medium was analyzed by high-pressure liquid chromatography (HPLC) using a Rainin HPLC equipped with a Microsorb-MVTM reverse-phase column (C_8; 5 μm; 300 Å; 4.6 × 250 mm). Products were separated with a gradient of 0.1 M triethylammonium acetate, pH 7.0, 95% to 5%, and acetonitrile 5% to 95% during 30 min and at a flow rate of 1 ml/min. Products were detected by reading absorbance at 505 nm. Peaks were identified by performing separate analyses with authentic samples of 5-chloromethylfluorescein and the glutathione adduct of 5-chloromethylfluorescein.

RESULTS

Selectivity of the Substrates for *lacZ*-Encoded Gene Activity

To investigate the selectivity of FDG, CMFDG, and C_{12}FDG for *lacZ*-encoded vs. endogenous lysosomal β-galactosidase activity, we incubated parallel cultures of NIH 3T3 cells, which express the lysosomal enzyme only, and CB2 cells, which express both enzyme activities, for 15 min in culture media at 37°C with 50 μM of the fluorogenic enzyme substrates. Immediately after incubation, samples were transferred to a melting ice bath, propidium iodide was added, and fluorescence of 10,000 cells was analyzed by flow cytometry. The results, as displayed in Table 1, demonstrate that FDG and CMFDG elicited a high level of fluorescence in CB2 cells, whereas C_{12}FDG gave rise to much lower fluorescence emission. For an indication of selectivity for *lacZ* vs. the lysosomal enzyme activity, the fluorescence ratio between CB2 and 3T3 cells was calculated. Both FDG and CMFDG gave a high fluorescence ratio (23.5 and 30.2, respectively), but fluorescence from C_{12}FDG was more biased toward the lysosomal enzyme activity (fluorescence ratio of 7.9). An attempt to enhance the

Table 1
Relative Fluorescence Intensity of Fluorogenic Enzyme Substrates in CB2 and NIH-3T3 Cells[a]

Substrate	Cells		
	CB2	NIH 3T3	Ratio CB2/NIH 3T3
FDG	100	4.3 ± 2.3	23.5 ± 4.4*
CMFDG	123.9 ± 8.1	4.1 ± 1.5	30.2 ± 3.1*
C_{12}FDG	14.2 ± 5.1	1.7 ± 1.1	7.9 ± 4.0

[a]Suspensions of CB2 and NIH 3T3 cells were incubated in culture medium for 15 min at 37°C with 50 μM of each of the substrates. Results are means and standard deviations from three independent experiments. The fluorescence obtained with FDG in CB2 cells is set to a value of 100 to allow for comparison among the experiments.

*CB2/NIH 3T3 ratio is significantly different from that of the C_{12}FDG samples at $P < 0.05$ in a two-sided Student's t test.

uptake of enzyme substrates by following the hypotonic-shock procedure, as described by Nolan and coworkers (11), failed to improve fluorescence generation by any of the three substrates. It should be noted that this procedure led to significant cell death in our cells (results not shown). Thus, whereas C_{12}FDG gave rise to little fluorescence and a lower selectivity toward the *lacZ*-encoded enzyme activity, both FDG and CMFDG showed a more favorable outcome in this experiment.

Lysosomal Alkalinization

Incubation with chloroquine leads to alkalinization of the lysosomes, such that the activity of α-galactosidase in cultured human skin fibroblasts is inhibited (3). Therefore, we analyzed the influence of chloroquine upon viability and fluorescence generation in NIH 3T3 cells exposed for 15 min at 37°C to 50 μM of CMFDG. Figure 1 shows that the fluorescence from lysosomal enzyme activity was lowered by chloroquine, depending on its concentration. Concomitantly, the number of viable cells decreased (Fig. 1). Hence, together with a possible reduction of interference from the lysosomal enzyme activity, chloroquine caused a significant loss of the number of viable cells available for study. Therefore, the possible use of chloroquine to increase the sensitivity of the assay for *lacZ*-encoded enzyme activity cannot be recommended.

Inhibition of Dye Efflux

As an alternative strategy to improve selectivity for the *lacZ*-encoded β-galactosidase, we investigated the inference of Jongkind and coworkers that fluorescent product of enzyme activity may be effluxed from the cells (7). We used probenecid and verapamil to probe for the influence of plasma membrane-resident drug efflux systems. In an explorative experiment, we incubated CB2 and NIH 3T3 cells with 50 μM each of FDG, CMFDG, or C_{12}FDG for 30 min at 37°C in the presence of either 1 mM probenecid or 100 μM verapamil. The ratio in fluorescence intensity between CB2 and NIH 3T3 cells, as outlined above, reflects the relative selectivity of the fluorogenic substrates for the *lacZ*-encoded vs. the lysosomal β-galactosidase activity. It is evident from Table 2 that the ratio in

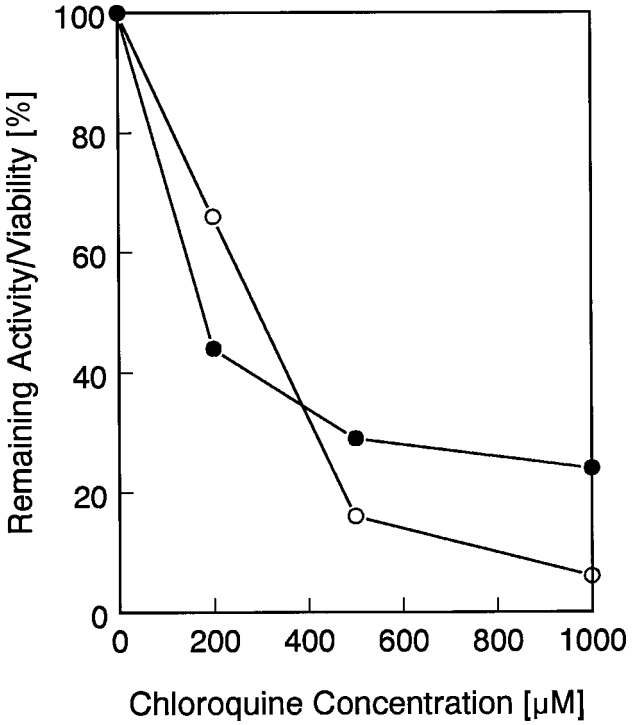

FIG. 1. Lysosomal β-galactosidase activity and cell viability after chloroquine treatment of NIH 3T3 cells. Cell suspensions were incubated for 15 min at 37°C to 50 μM of CMFDG and the indicated concentration of chloroquine. Viability is the fraction of cells that excluded propidium iodide after the incubation. Open circles, viability data; solid circles, remaining enzyme activity. Data are mean of three independent experiments.

fluorescence intensity between CB2 and NIH 3T3 cells incubated with FDG and CMFDG is similar, whereas C_{12}FDG gave rise to less selectivity for the *lacZ*-encoded enzyme. Exposure to probenecid slightly improved the selectivity for the *lacZ*-encoded enzyme of all three enzyme substrates. Verapamil, on the other hand, improved selectivity for the *lacZ*-encoded enzyme of CMFDG strongly but improved less for FDG (Table 2). Thus, this preliminary experiment suggests that inhibition of drug efflux with verapamil may enhance retention of fluorescence from FDG and CMFDG. In addition, verapamil may enhance the selectivity of CMFDG toward the *lacZ*-encoded enzyme.

To determine the optimal verapamil concentration required to improve the selectivity of FDG, CMFDG, and C_{12}FDG toward the *lacZ*-encoded enzyme, we incubated CB2 and NIH 3T3 cells for 15 min at 37°C with the fluorogenic substrates and a series of verapamil concentrations. Figure 2 shows that a gradual rise in fluorescence generation by CB2 cells incubated with FDG or CMFDG and increasing concentrations of verapamil were obtained. In NIH 3T3 cells, the fluorescence with the same fluorogenic substrates did not rise with increasing verapamil concentrations. In contrast to the results with FDG and CMFDG, C_{12}FDG afforded no significant increase in fluorescence with rising verapamil concentrations (Fig. 2). This

Table 2
Influence of Inhibitors of Drug Efflux on the Level of Fluorescence From Fluorogenic β-Galactosidase Substrates[a]

Dye	Treatment	Fluorescence intensity: CB2 cells	NIH 3T3 cells	Ratio CB2/NIH 3T3	Improvement in ratio
FDG	Control	516	67	7.7	
	Probenicid	1,147	132	8.7	×1.1
	Verapamil	889	57	15.6	×2.0
CMFDG	Control	577	77	7.5	
	Probenicid	1,194	118	10.1	×1.3
	Verapamil	1,638	75	21.8	×2.9
C_{12}FDG	Control	80	14	5.7	
	Probenicid	130	18	7.2	×1.3
	Verapamil	84	14	6.0	×1.1

[a]CB2 and NIH 3T3 cells were incubated with 50 μM of each FDG, CMFDG, and C_{12}FDG for 15 min at 37°C in the presence of either 1 mM probenicid or 100 μM verapamil. The results are from a typical experiment out of a series of three.

result is consistent with our findings, which are described above (Table 2). Verapamil at concentrations up to 200 μM did not affect cell viability, but, at 500 μM, significant cell death was observed (results not shown). We conclude that a verapamil concentration of 200 μM maximally enhances resolution between *lacZ*-expressing (CB2) and nonexpressing (NIH 3T3) cells.

Because CMFDG gave the most *lacZ*-selective signal and the strongest verapamil responsive fluorescence, we chose to investigate further the use of this fluorogenic β-galactosidase substrate. A drawback of many FDG-based protocols for *lacZ*-monitoring is the limited time of incubation during which the signal remains linear (1,11). The sensitivity of the assay could be improved if the time interval of enzyme activity monitoring could be extended. This requires a linear response with time. Therefore, we investigated a time course of fluorescence development of CB2 and NIH 3T3 cells incubated continuously with 50 μM CMFDG in the presence or absence of 200 μM verapamil. Fluorescence from CB2 cells incubated with CMFDG, as is evident from Figure 3, increased linearly with time, and a steeper slope was found in the presence of verapamil. The fluorescence of NIH 3T3 cells incubated with CMFDG increased marginally with time, and this increase was not significantly enhanced by the presence of verapamil (Fig. 3). Thus, the resolution between *lacZ*-encoded and endogenous β-galactosidase activity increased linearly with incubation time. This characteristic will allow investigators to tune their experiments to a desired level of fluorescence intensity and resolution.

Apart from extending the time interval for monitoring *lacZ*-encoded β-galactosidase activity, one could potentially enhance the sensitivity of the assay by incubation with higher substrate concentrations. Again, this requires a linear response of cellular fluorescence. A linear fluorescence response, as is depicted in Figure 4, was found in CB2 cells incubated for 15 min at 37°C with increasing concentrations of CMFDG in the presence of 200 μM of verapamil. When verapamil was omitted from the incubation, the fluorescence response leveled off at CMFDG concentrations above 50 μM. The amount of fluorescence obtained with NIH 3T3 cells increased to a limited extent with rising CMFDG concentrations. Thus, by increasing the CMFDG concentration in the incubation system and with the addition of verapamil, one can improve the resolution between *lacZ*-encoded and endogenous β-galactosidase activity.

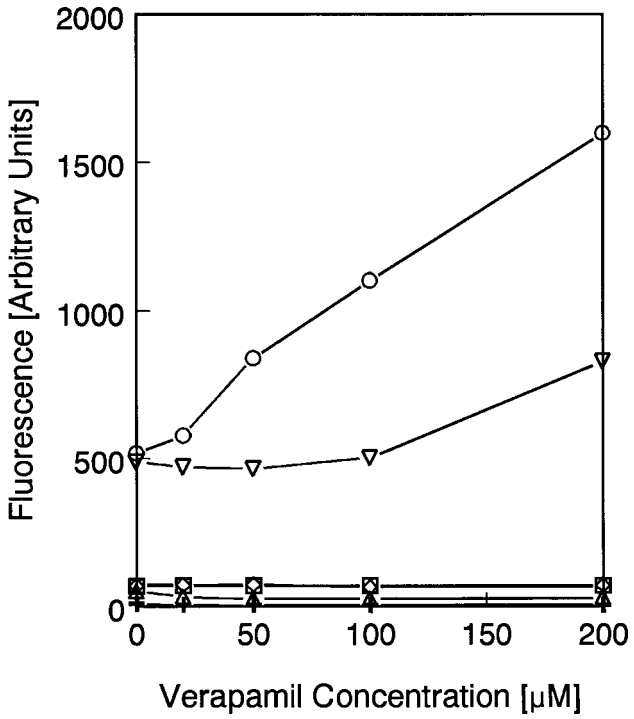

FIG. 2. Fluorescence generated with fluorogenic β-galactosidase substrates as a function of verapamil concentration. Suspensions of CB2 and NIH 3T3 cells were incubated for 15 min at 37°C with 50μM of the fluorogenic β-galactosidase substrates and a series of verapamil concentrations. Circles, CMFDG; inverted triangles, FDG; squares, C_{12}FDG incubated with CB2 cells. Diamonds, CMFDG; upright triangles, FDG; plus signs, C_{12}FDG incubated with NIH 3T3 cells. Data are from a typical experiment out of a series of three.

Because CMFDG contains two functional groups, a pair of galactose residues and a chloromethyl moiety, it can be envisaged that several fluorescent products may result from metabolism of CMFDG. Thus, either the substrate is only hydrolyzed by β-galactosidase to yield 5-chloromethylfluorescein or the chloromethyl moiety may react with an intracellular thiol group, leading to the formation of a protein or a glutathione adduct. Reaction of the substrate with a thiol group without hydrolysis of the galactosides will lead to the formation of a nonfluorescent adduct, which cannot be detected in our assay. In order to discriminate between these possibilities and to determine the mechanism by which verapamil enhanced the generation of fluorescence from metabolic conversion of CMFDG, we analyzed the fluorescein-related products that were effluxed from CB2 cells during a 15 min incubation with 50 μM of substrate. Table 3 shows that 79.2 pmoles of 5-chloromethylfluorescein and 1.67 pmoles of the glutathi-

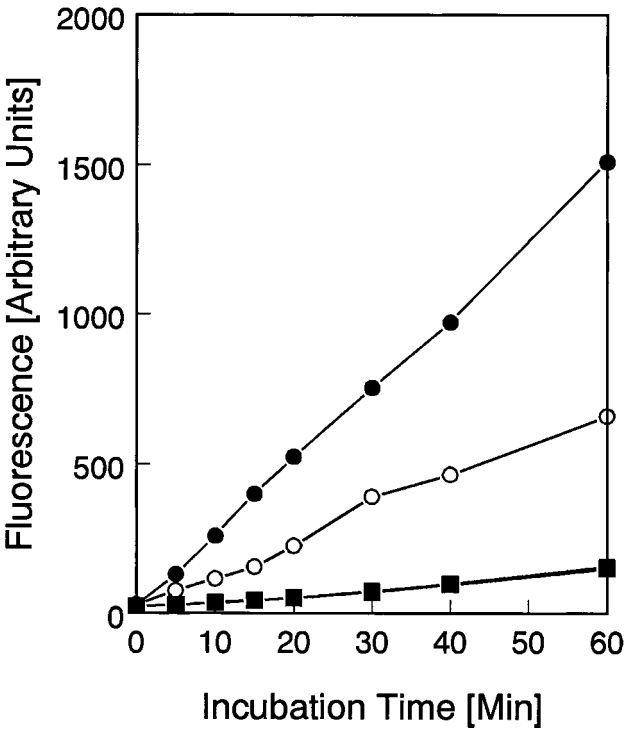

FIG. 3. Time course of fluorescence generation by suspensions of CB2 and NIH 3T3 cells incubated at 37°C with 50 μM of CMFDG in the absence or presence of 200 μM verapamil. Solid circles, CB2 cells in the presence of verapamil; open circles, CB2 cells in the absence of verapamil; solid squares, NIH 3T3 cells in the presence or absence of verapamil (superimposed data points). Data are from a typical experiment out of a series of three.

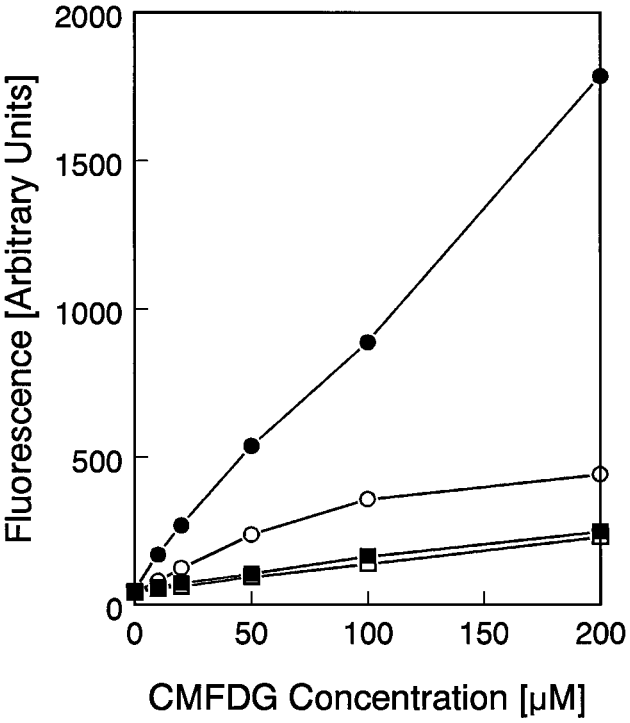

FIG. 4. Fluorescence generation as a function of CMFDG concentration by suspensions of CB2 and NIH 3T3 cells in the absence and the presence of 200 μM verapamil. Solid circles, CB2 cells in the presence of verapamil; open circles, CB2 cells in the absence of verapamil; solid squares, NIH 3T3 cells in the presence of verapamil; open squares, NIH 3T3 cells in the absence of verapamil. Data are from a typical experiment out of a series of three.

one adduct of 5-chloromethylfluorescein were effluxed by 1×10^6 cells in 15 min. Coincubation with 200 μM verapamil reduced the amount of effluxed 5-chloromethylfluorescein to 25.4 pmoles (68% reduction) and reduced the amount of effluxed glutathione adduct of 5-chloromethylfluorescein to 0.89 pmoles (47% reduction). Verapamil apparently exhibits some degree of selectivity for the efflux of 5-chloromethylfluorescein, although efflux of the glutathione adduct is also affected by the drug.

Table 3
High-Pressure Liquid Chromatography Analysis of Products of CMFDG Hydrolysis Effluxed by CB2 Cells[a]

Effluxed product	Incubation (pmoles/10^6 cells/15 min) Control	200 μM Verapamil	Ratio of sample with verapamil relative to control
5-Chloromethyl-fluorescein	79.2 ± 3.1	25.4 ± 1.5	0.32
Glutathione adduct	1.7 ± 0.2	0.9 ± 0.2	0.53

[a]Results are means and standard deviations from three independent experiments, as described in Materials and Methods.

DISCUSSION

In this study, we compared the fluorescence generated with three fluorogenic β-galactosidase substrates in *lacZ*-expressing (CB2) and nonexpressing NIH 3T3 cells. We found the most lipophilic of the three substrates (C_{12}FDG) to be the most selective for lysosomal β-galactosidase. This can be explained easily: Lysosomal β-galactosidase resides in the lysosomal membrane in a complex that is also comprised of neuraminidase and a ''protective protein'' (2,6). The C_{12}FDG molecule is likely to insert itself into the lysosomal membrane, whereupon the galactosides can be removed and fluorochromasia results. The extent of lysosomal cleavage of C_{12}FDG is likely to be underestimated, because the pKa of fluorescein is around 6.5, whereas the lysosomal pH is about 4.5. A fluorophore with a more acidic pKa may more accurately record the lysosomal β-galactosidase activity. The contribution of lysosomal enzyme activity to total fluorochromasia is not relevant in cell systems devoid of lysosomes (8,20); therefore, it has not been dealt with in previous studies.

The more hydrophilic β-galactosidase substrates, FDG and CMFDG, are more selective for the cytosolic *lacZ*-encoded enzyme (Table 1). We sought to improve the selectivity of FDG and CMFDG toward the cytosolic enzyme further by incubation with chloroquine to shift the lysosomal pH toward neutral values. Although this treatment did result in lowering lysosomal enzyme-dependent generation of fluorescence, it also lead to significant cell loss (Fig. 1). Jongkind and coworkers suggested that fluorescent products resulting from cleav-

age of FDG may leak out of the cell (7). Inhibition of this leakage may constitute another way of improving the selectivity of the fluorogenic substrates toward the *lacZ*-encoded enzyme. Coincubation with verapamil proved to be successful, in that a threefold enhancement of resolution between *lacZ*-encoded and lysosomal enzyme activity was found with CMFDG (Figs. 3, 4). In addition, a linear increase of fluorescence generation was observed. Thus, the CMFDG-based assay for *lacZ*-encoded β-galactosidase activity became amenable to longer incubation intervals, and, as a result, its sensitivity is likely to be higher than in the method followed by Brugstugun and coworkers, who used microinjection of CMFDG (1).

Another interesting result from our study concerns the mechanism by which the fluorescent products of FDG and CMFDG hydrolysis are effluxed from the cell. This is not likely to be due to activity of the multidrug resistance protein (MRP), because fluorescence accumulation with FDG and CMFDG showed little sensitivity toward probenecid, an inhibitor of MRP-mediated drug export (4). Also, a possible role of glutathione conjugation in the efflux of CMFDG seems to be unlikely, because export of the glutathione adduct of monochlorobimane appears to be mediated by a mechanism that is sensitive toward probenecid but not toward verapamil (14). The results of our HPLC analyses, which demonstrate that most of the effluxed product is identical with 5-chloromethylfluorescein, further support this view. Therefore, the effect of verapamil on the efflux of fluorescent products from the cell can apparently be accounted for by a reduction of 5-chloromethylfluorescein efflux. It is unknown whether the MDR-1-encoded drug efflux system (9) may be involved in the efflux of 5-chloromethylfluorescein or whether another, not yet characterized system may be responsible for this phenomenon. Regardless of the molecular mechanism involved, inclusion of verapamil in the incubation system may improve assays for *lacZ*-encoded β-galactosidase activity based on CMFDG.

ACKNOWLEDGMENTS

We are indebted to Dr. Kyle R. Gee (Molecular Probes, Inc.) for making the authentic samples of 5-chloromethylfluorescein and its glutathione adduct available to us.

LITERATURE CITED

1. Brugstugun OT, Mellgren G, Gjertsen BT, Bjerkvig R, Dóskeland SO: Sensitive and rapid detection of β-galactosidase expression in intact cells by microinjection of fluorescent substrate. Exp Cell Res 219:372–378, 1995.
2. D'Azzo A, Hoogeveen A, Reuser AJ, Robinson D, Galjaard H: Molecular defect in combined β-galactosidase and neuraminidase deficiency in man. Proc Natl Acad Sci USA 79:4535–4539, 1982.
3. De Groot PG, Oude Elferink R, Hollemans M, Strijkland A, Westerveld A, Meera Khan P, Tager JM: Inactivation by chloroquine of α-galactosidase in cultured human skin fibroblasts. Exp Cell Res 136:327–333, 1981.
4. Feller N, Broxterman HJ, Währer DCR, Pinedo HM: ATP-dependent efflux of calcein by the multidrug resistance protein (MRP): No inhibition by intracellular glutathione depletion. FEBS Lett 368:385–388, 1995.
5. Hofmann J, Sernetz M: A kinetic study on the enzymatic hydrolysis of fluoresceindiacetate and fluorescein-di-β-D-galactopyranoside. Anal Biochem 131:180–186, 1983.
6. Hoogeveen AT, Verheijen FW, Galjaard H: The relation between human lysosomal β-galactosidase and its protective protein. J Biol Chem 258:12143–12146, 1983.
7. Jongkind JF, Verkerk AF, Sernetz M: Detection of acid-β-galactosidase activity in viable human fibroblasts by flow cytometry. Cytometry 7:463–466, 1986.
8. Jasin M, Zalamea P: Analysis of *Escherichia coli* β-galactosidase expression in transgenic mice by flow cytometry of sperm. Proc Natl Acad Sci USA 89:10681–10685, 1992.
9. Kessel D, Beck WT, Kukuruga D, Schulz V: Characterization of multidrug resistance by fluorescent dyes. Cancer Res 51:4665–4670, 1991.
10. Mavilio F, Ferrari G, Rossini S, Nobili N, Bonini C, Casorati G, Traversari C, Bordignon C: Peripheral blood lymphocytes as target cells of retroviral vector-mediated gene transfer. Blood 83:1988–1997, 1994.
11. Nolan GP, Fiering S, Nicolas J-F, Herzenberg LA: Fluorescence-activated cell analysis and sorting of viable mammalian cells based on β-D-galactosidase activity after transduction of *Escherichia coli lacZ*. Proc Natl Acad Sci USA 85:2603–2607, 1988.
12. Pilon M, Gullberg M, Lundgren E: Transient expression of the CD2 cell surface antigen as a sortable marker to monitor high frequency transfection of human primary B cells. J Immunol 146:1047–1051, 1991.
13. Poot M, Kavanagh TJ, Kang HC, Haugland RP, Rabinovitch PS: Flow cytometric analysis of cell cycle-dependent changes in cell thiol level by combining a new laser dye with Hoechst 33342. Cytometry 12:184–187, 1991.
14. Poot M, Hudson FN, Grossmann A, Rabinovitch PS, Kavanagh TJ: Probenecid inhibition of fluorescence extrusion after MCB-staining of rat-1 fibroblasts. Cytometry 23:78–81, 1996.
15. Price J, Turner D, Cepko C: Lineage analysis in the vertebrate nervous system by retrovirus-mediated gene transfer. Proc Natl Acad Sci USA 84:156–160, 1987.
16. Rose M, Casabadan MJ, Botstein D: Yeast genes fused to β-galactosidase in *Escherichia coli* can be expressed normally in yeast. Proc Natl Acad Sci USA 78:2460–2464, 1981.
17. Rotman B, Zderik JA, Edelstein M: Fluorogenic substrates for β-D-galactosidases and phosphatases derived from fluorescein (3,6-dihydroxyfluoran) and its monomethyl ether. Proc Natl Acad Sci USA 50:1–6, 1963.
18. Wittrup KD, Bailey JE: A single-cell assay of β-galactosidase activity in *Saccharomyces cerevisiae*. Cytometry 9:394–404, 1988.
19. Yeh E, Gustafson K, Bouillianne GL: Green fluorescent protein as a vital marker and reporter of gene expression in *Drosophila*. Proc Natl Acad Sci USA 92:7036–7040, 1995.
20. Zhang Y-Z, Naleway JJ, Larison KD, Huang Z, Haugland RP: Detecting *lacZ* gene expression in living cells with new lipophilic, fluorogenic β-galactosidase substrates. FASEB J 5:3108–3113, 1991.