Creation of Polarized Cells Coexpressing CYP3A4, NADPH Cytochrome P450 Reductase and MDR1/P-glycoprotein

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Purpose. To develop model polarized cell systems expressing cytochrome P4503A4, NADPH P450 reductase, and P-glycoprotein (Pgp).
Methods. LLC-PK1 and derivative L-MDR1 cells stably expressing Pgp, the product of the multidrug resistance gene (*MDR1*), were transfected stably using either a mammalian neomycin selectable expression vector (CYP3A4-Neo) or an episomal vector based on Epstein–Barr virus (CYP3A4-Hygro). These CYP3A4 expressing cells were compared with LLC-PK1, L-MDR1, or Caco-2 cells transduced with Adenovirus-3A4 vector (Ad3A4) with or without simultaneous Adenovirus-P450 Reductase (AdRed) transduction. Cells were characterized for expression of CYP3A4 protein and CYP3A4 mediated metabolism towards midazolam and testosterone. Analysis of membrane integrity and drug transport assays were performed to determine whether infection with recombinant Ad3A4 ± AdRed affected Pgp function.
Results. The rank order of optimal CYP3A4 expression and activities in LLC-PK1 and L-MDR1 cells from highest to lowest was cells co-transduced with Ad3A4 plus AdRed >> Ad3A4 >>> CYP3A4-Hygro > CYP3A4-Neo. Similarly, coexpression of Ad3A4 plus AdRed led to enhanced CYP3A4 mediated metabolism in Caco-2 cells over cells with Ad3A4 alone. Incubation of transwell cultured cells expressing Ad3A4/AdRed with midazolam led to readily detectable metabolite in the medium. In microsomes from Caco-2 and LLC-PK1 cells, each co-transduced with Ad3A4/AdRed, Vmax values for testosterone 6β-hydroxylase activity ranged from 414 to 1350 pmoles/min/mg, respectively. For either Caco-2 or LLC-MDR1 cells, TEER values and the rate of apical to basal and basal to apical transport of vinblastine or digoxin were similar in cells with and without Ad3A4/Red transduction.
Conclusions. Polarized cellular systems coexpressing Ad3A4, AdRed, and the MDR1/Pgp transporter were developed and characterized. The results document the utility of these polarized model systems for simultaneous drug transport/drug metabolism studies. Since the experimental approach can be adapted to study the interplay of multiple enzyme/transporting systems, it may find significant application as a screening tool for the pharmaceutical industry and as a more basic research tool to study the kinetics of intestinal drug bioavailability.

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ABBREVATIONS: P450 reductase, NADPH cytochrome P450 reductase; Ad, adenovirus; P-glycoprotein, Pgp.

KEY WORDS: CYP3A4; Pgp; Caco-2; LLC-PK1.

INTRODUCTION

A variety of approaches have been used to develop cellular model systems that express cytochromes P450 (CYPs). These include heterologous expression in mammalian cells using Vaccinia virus, Epstein-Barr virus, and retrovirus vectors, and introduction of transgenes containing selectable marker genes (reviewed in (1,2)). The level of functional CYP expression varies considerably between the model systems (2–4) and the utility of the models varies significantly depending on the host cell. Despite these advances, there has been little work on introduction of CYPs into polarized cells or cells containing drug transporters.

However, we have recently demonstrated that there is a dynamic interplay between drug transporting and drug metabolizing systems at the level of CYP regulation. We demonstrated that P-glycoprotein (Pgp) influenced the intracellular concentration of a CYP3A inducer, rifampicin, and thus a pharmacological action of this drug in the cell, namely the magnitude of CYP3A induction (5). We further hypothesized (5) that for drugs that are substrates for both Pgp and CYP3A4, Pgp could also influence the rate and extent of CYP3A4 mediated metabolism. However, most of the cellular systems currently described are not suited to transport experiments examining vectoral movement of substrates because the host cells do not polarize in culture. Further, the majority of host cells (e.g., V79, SF21, lymphoblastoid) do not express drug transporters. Towards the goal of elucidating a functional interaction between Pgp mediated efflux and CYP3A4 metabolism, we generated cell lines that expressed either singly or in combination CYP3A4 and/or Pgp.

We chose two different cellular models. Caco-2 cells were selected because they express Pgp, polarize in culture, and retain functional characteristics of human small intestine. These features make them suitable for routine screening for intestinal permeability and oral drug bioavailability of compounds (6). However, it has been clearly demonstrated that an important component of first pass drug metabolism is intestinal CYP3A4 (7), an enzyme that is expressed weakly, if at all, in standard Caco-2 cells (3).

LLC-PK1 and derivative cell lines stably expressing human Pgp were chosen as recipients for CYP3A4 expression vectors because this cell polarizes in culture, and has been used extensively to characterize transport function of Pgp (8). Moreover, additional cell lines ectopically expressing other drug efflux transporters (e.g., the multidrug resistance associated proteins MRP1 and MRP2 (9,10), and Sister P-glycoprotein (11)) have been created using this same LLC-PK1 host. Thus, knowledge of the suitability of LLC-PK1 cells for interaction studies between CYP3A4 and Pgp could readily be extended to other drug metabolizing enzymes and other drug uptake and efflux transporters.

We compared mammalian expression vector systems to determine which gave optimal CYP3A4 protein expression and enzymatic activity in Caco-2 and LLC-PK1 cells. Stable cell lines were generated using vectors that integrate into genomic DNA and those that are episomally maintained. These were compared with cells transduced with a novel adenoviral vector

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(AdV) expressing CYP3A4 (Ad3A4) or co-transduced with a second adenoviral vector expressing P450 reductase (AdRed).

MATERIALS AND METHODS

Materials

Recombinant human IL-1β was purchased from R & D Systems (Minneapolis, MN). Testosterone and 6β-hydroxytestosterone were obtained from Steraloids Inc. (Wilton, NH). [^{3}H]vinblastine sulphate (11.7 Ci/mmol) from Moravek (Brea, CA), and [^{3}H]digoxin (19 Ci/mmol) was from NEN Life Sciences (Boston, MA).

Cell Culture and Transport Assays

LLC-PK1 and derivative cell lines containing human *MDR1* (L-MDR1) were obtained from Dr. Alfred Schinkel (The Netherlands Cancer Institute, Amsterdam, The Netherlands) (8). Caco-2 cells were obtained from Dr. Richard Kim (Vanderbilt University, Nashville, TN).

Transport assays were performed exactly as previously described (8). Briefly, LLC-PK1 cells were plated on day 0 at 1×10^6 cells/12 mm well. On day 1 medium was changed and cells were transduced with AdV as described below. On day 4 cells were washed and at time 0 the assay started by adding radiolabeled drug to either the apical or basal compartment. At 1, 2, 3, and 4 hr 50 μl aliquots were sampled from the opposite compartment, counted, and expressed as the % radioactivity appearing in the opposite compartment relative to radioactivity added at time 0. The integrity/barrier function of the cell monolayers was determined by routinely measuring before the experiment the transepithelial electrical resistance (TEER). The radiolabeled drug was always added at a 2 μM final concentration containing ~0.25 μCi/ml. Caco-2 cells were plated at 1×10^6 cells/12 mm well on day 1, transduced on day 15 with AdV and transport assessed on day 20.

CYP3A4-Hygro

CYP3A4 (from Frank Gonzalez, NIH, Bethesda, MD) was subcloned into RSV promoter expression vector pRep10 (In Vitrogen, Carlsbad, CA).

CYP3A4-Neo

CYP3A4 was cloned into pcDNA3.1/myc-His (In Vitrogen) to generate CYP3A4-Neo.

Generating Stable Cell Lines

LLC-PK1 or L-MDR1 cells were transfected by calcium phosphate co-precipitation with DNA and selected for plasmid expression with G418 (600 μg/ml) or hygromycin (660 U/ml).

Adenoviral Transduction of Cell Lines

For most experiments LLC-PK1 or Caco-2 cells were plated at 1×10^6 cells/35 mm well on day 1. On day 2 cells were transduced with various AdV MOIs in 0.5 ml of serum free medium for 90 min. Then 1.5 ml of serum containing medium was added. On day 3 medium was removed and fresh serum containing medium added. On day 5 cells were assayed for CYP3A protein and activities.

Preparation of Adenovirus

AdGFP (adeno-green fluorescent protein) (12). Ad3A4 and AdRed: The AdRSV3A4 and AdRSVRed (referred to hereafter as Ad3A4 and AdRed) were kindly provided by Genotherapeutics, Inc. (Memphis, TN). A human CYP3A4 cDNA was subcloned under the control of a RSV promoter into an adenoviral shuttle vector. The resultant adenoviral shuttle vector was cotransfected into 293 cells with pJM17 (13), an adenoviral type 5 genome plasmid, by calcium phosphate method. Individual plaques were screened for recombinant AdRSV3A4 by PCR using specific primers for both the RSV promoter and 3A4 cDNA sequences. Adenovirus expressing human NADPH P450 reductase (AdRSVRed) was similarly constructed. Single viral clones were propagated in 293 cells. The culture medium of the 293 cells showing the completed cytopathic effect (CPE) was collected, and the adenovirus was purified and concentrated by twice $CsCl_2$ gradient ultracentrifugation. The viral titration and transduction were performed as previously described (14).

Preparation of Cell Fractions

Cells were scraped, washed and pelleted, pellets resuspended in microsomal storage buffer (100 mM potassium phosphate, pH 7.4, 1.0 mM EDTA, 20% glycerol, 1 mM dithiothreiotol, 20 μM butylated hydroxytoluene, 2 mM phenylmethylsulfonyl fluoride) [MSB]) and sonicated to yield cell lysates. Microsomes were prepared by high speed centrifugation of lysate at 105,000 × g for 1 hr and the microsomal pellet resuspended in MSB.

Immunoblot Analysis

10 μg of cell lysate or cell microsomes was separated on 10% slab polyacrylamide gels and immunoblotted using the following antibodies: monoclonal anti-CYP3A4 K03 (15) or rabbit anti-rat P450 reductase (from Dr. Ken Thummel, Univ. Washington, Seattle, WA) and by appropriate secondary antibodies followed by ECL detection (Amersham).

Midazolam Hydroxylation in Cell Lysates and Microsomes

4-hydroxymidazolam and 1′-hydroxymidazolam were assayed as previously described in cell *microsomes* (0.2 mg/incubate) at 60 μM midazolam (5). Total cell *lysates* (1.5–4.2 mg protein/replicate) were preincubated for 10 min with 60 μM midazolam. All reactions were initiated by addition of $1/10^{th}$ volume of an NADPH-generating system (10 Units/ml isocitrate dehydrogenase, 50 mM isocitrate, 10 mM sodium NADP, and 50 mM magnesium chloride). Lysates were incubated at 37°C for 30 min and the reaction stopped by addition of 5 ml t-butylether. Samples were mixed, centrifuged at 800 × g and the lower aqueous phase removed by freezing in a dry ice/acetone bath. The ether was evaporated under a stream of nitrogen, the residue reconstituted in 200 μl of mobile phase, and 100 μl was injected onto the HPLC. Enzyme activities were expressed as nmol of product/mg protein/hr.

Midazolam Hydroxylation in Monolayer Cultures

Caco-2 or LLC-PK1 cells transduced with Ad3A4 and AdRed were incubated with medium supplemented with 20 or 10% FBS, respectively, and containing 4–60 μM midazolam for 4 hr. One ml of medium was removed, and extracted as above with 5 ml of t-butylether and assayed by HPLC.

Testosterone 6β-Hydroxylase Activities

CYP3A4 activity was determined by examining the rate of 6β-hydroxytestosterone formation of microsomal fractions from each cell line. Briefly, a 0.25 ml reaction mixture containing 1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride (NADPH generating solution), 0.5 mg/ml protein, 100 mM potassium phosphate buffer (pH 7.4), and 12.5 μM to 200 μM final testosterone concentration was added together (2,3). The reaction was initiated by addition of the NADPH generating solution, run for 10 min at 37°C, stopped with 125 μl acetonitrile spiked with 0.04 mM (5 nmol) dexamethasone. The mix was extracted with 1.5 ml of ethyl acetate, centrifuged (600 × g), the upper organic layer removed, evaporated to dryness, the residue dissolved in 200 μl of mobile phase and a 100-μl injected on a C_{18} reversed phase column (Waters Nova-Pak 3.9 × 150 mm, 3 μm particle size, Waters Corp., Milford, MA) preceded by a C_{18} guard column (Waters Corp.). The peak height of 6β-hydroxytestosterone, dexamethasone and testosterone was measured using commercially available software (Millenium, Waters Corp.). Kinetic parameters describing the rate of metabolism were determined from plots of the initial rate of 6β-hydroxytestosterone formation versus testosterone concentration. Specifically, the maximal rate of 6β-hydroxytestosterone formation V_{max} and Michaelis-Menten constant K_m were calculated using a FORTRAN subroutine and WinNonlin computer program (Pharsight Corporation, Mountain View, CA).

RESULTS

We compared several techniques for introducing CYP3A4 into cell lines to determine the method giving maximal CYP3A4 expression. The first approach was to use recombinant adenovirus (AdV) vectors. Although AdV will transduce almost every cell type, the efficiency of this transduction varies between cell lines (16). Therefore we first characterized the competency of AdV transduction of our host cells. LLC-PK1, L-MDR1 and Caco-2 cells were infected with different multiplicities of infection (MOI) of an Ad-green fluorescent protein (GFP) vector. The proportion of GFP-positive cells at 48 hrs post-transduction was measured by fluorescent activated cell sorting (FACS) analysis. With increasing levels of AdGFP there was a dose-dependent increase in the amount of recombinant GFP cellular fluorescence (Fig. 1). The percentage of GFP-positive cells was 97%, 87% and 99% in LLC-PK1, Caco-2 and L-MDR1 cells, respectively at an MOI of 50 two days after infection with AdGFP (Fig. 1A). There was a dose-dependent increase in GFP expression between MOI of 1-100 (Fig. 1B), but an MOI of 500 resulted in lower GFP expression in LLC-PK1 and L-MDR1 cells most likely reflecting the higher percentage of dead cells at this amount of AdV (not shown). We consistently observed that the LLC-PK1 cells transduced with a much higher

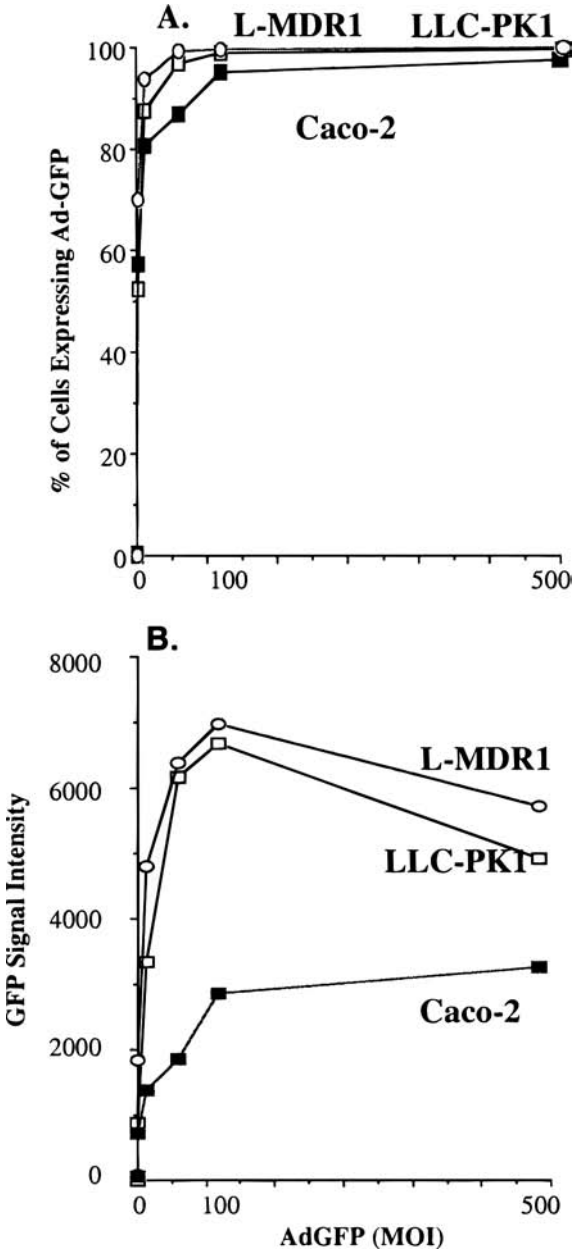

Fig. 1. GFP expression in cells infected by an AdGFP recombinant virus. LLC-PK1, L-MDR1, and Caco-2 cells were transduced with the indicated MOI of AdGFP and harvested two days later and (A) the percentage of cells expressing AdGFP and (B) the maximum fluorescent intensity was analyzed by FACS analysis.

efficiency than Caco-2 (Fig. 1B). These transduction efficiencies were highly reproducible from experiment-to-experiment.

We next determined the MOI necessary to achieve maximum expression of CYP3A4 in Ad3A4 transduced cells. A dose-dependent increase in CYP3A4 protein was observed from an MOI of 10 to 1000 (Fig. 2A), with very high cellular toxicity evident at 1000 (not shown). Consistent with the findings for Ad-GFP, expression of CYP3A4 was higher in the LLC-PK1 compared to Caco-2 cells. Expression of CYP3A4 protein was at least 22.6-fold higher in Ad3A4 transduced LLC-PK1 cells compared with Caco-2 cells cultured on matrigel and treated

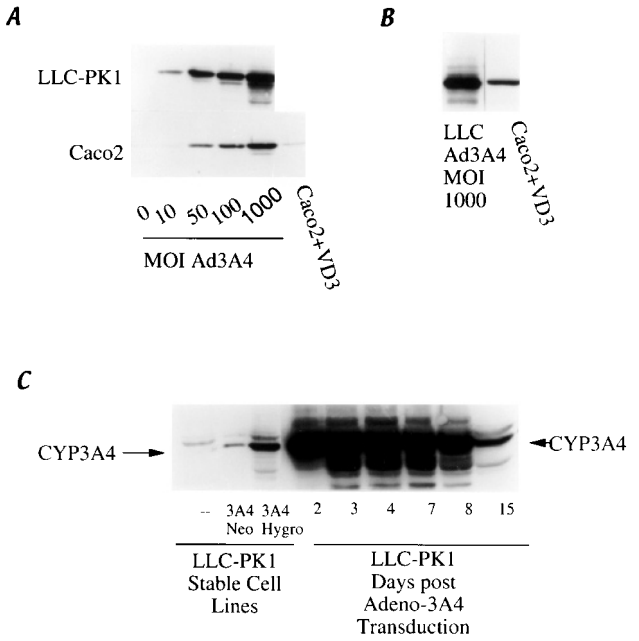

Fig. 2. CYP3A4 expression in cells infected with Ad3A4. (A) LLC-PK1 and Caco-2 were transduced with the indicated MOI of Ad3A4 and 48 hrs later 10 μg of total cell lysate was resolved on 12.5% PAGE and analyzed on immunoblot with anti-CYP3A4 IgG. 10 μg of lysate from Caco-2 cells treated with 1,25-dihydroxyvitamin D3 (VD3) were run simultaneously. (B) Side-by-side comparison of 10 μgs lysate from LLC-PK1 cells transduced with 1000 MOI Ad3A4 and Caco-2 cells cultured with 1,25-dihydroxyvitamin D3 analyzed as in (A). (C) 10 μg of total cell lysate from LLC-PK1 cells or LLC-PK1 cells stably transfected with CYP3A4-Neo or CYP3A4-EBV (Hygro) or transduced with Ad3A4 on day zero and cells analyzed on days 2–15 thereafter were subjected to 12.5% PAGE followed by immunoblotting with anti-CYP3A IgG.

with 0.25 μM 1,25-dihydroxyvitamin D3 for two weeks (a kind gift of Dr. Paul Watkins) (Figs. 2A,B) to induce endogenous CYP3A4 expression (3).

We next compared CYP3A4 expression in clonal cell lines created using two other vectors. CYP3A4 neo utilized an expression vector containing a cytomegalovirus enhancer/promoter region that drives CYP3A4 transcription and also contains the selection marker encoding the gene for neomycin phosphotransferase that permits cell growth in G418 containing medium. CYP3A4-Neo was transfected into LLC-PK1 cells and individual G418 resistant clones selected. The advantage of this approach was stable integration of the plasmid. The third approach was to clone CYP3A4 into an Epstein-Barr Virus (EBV) based expression vector that is maintained episomally and contains the gene conferring resistance to hygromycin B. Although this plasmid remains episomal, up to several hundred copies per cell of the vector can be maintained resulting in robust expression of the cDNA. Following immunoblot screening for CYP3A4 protein expression the CYP3A4-Neo and CYP3A4-Hygro clones with the highest level of CYP3A4 were selected and compared to LLC-PK1 cells transduced with Ad3A4 (Fig. 2C). CYP3A4 expression was highest in Ad3A4 LLC-PK1 cells compared to either LLC-3A4-Hygro or LLC-3A4-Neo (Fig. 2C). Indeed, CYP3A4 expression was at least 24-fold higher in LLC-Ad3A4 cells compared to LLC-CYP3A4-Neo. Although the LLC-3A4-Hygro cells remained resistant to hygromycin B over many passages, expression of CYP3A4 was lost/silenced within 15 passages. This observation is similar to loss of CYP3A4 expression in Caco-2 cells transfected with a similar CYP3A4-EBV vector approach (4). Finally, we examined how long the Ad3A4 expression persisted in the cells. Subconfluent cultures were transduced with Ad3A4. CYP3A4 expression remained high and persistent for at least 15 days in subconfluent cells transduced with Ad3A4 (Fig. 2C). To determine whether the CYP3A4 protein produced by Ad3A4 was catalytically active we measured the formation of 1′-and 4-hydroxymidazolam, a CYP3A specific reaction (17). Analysis of cell lysates from Ad3A4 transduced cells revealed a dose-dependent increase in midazolam hydroxylase activity between an Ad3A4 MOI of 10 and 1000 MOI in both LLC-PK1 and Caco-2 cells (not shown).

Suboptimal expression of NADPH P450 reductase in many cell lines, including Caco-2 (18) can limit maximal CYP catalytic activities. Therefore we determined whether stable transfection of P450 reductase-Neo or co-transduction of a recombinant-P450 reductase adenovirus (AdRed) would enhance CYP3A4 activity. Transduction of 1-25 MOI of AdRed resulted in a dose-dependent increase in P450 reductase protein expression (Fig. 3A). P450 reductase was also well expressed in LLC-Reductase-Neo clones (Fig. 3B). Analysis of cell lysates showed that in LLC-PK1 or Caco-2 cells transduced with

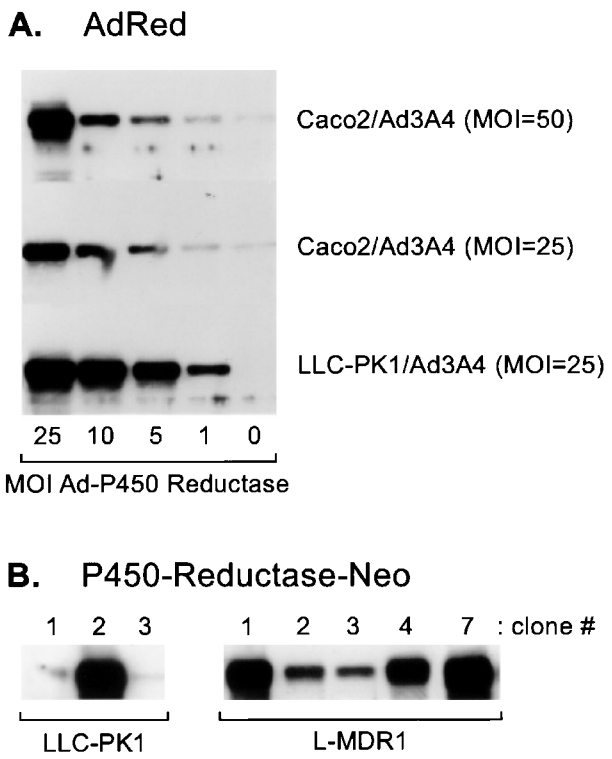

Fig. 3. Expression of ectopically expressed NADPH P450 reductase. (A) LLC-PK1 and Caco-2 cells were transduced with Ad3A4 at an MOI of 25 or 50 with or without (0) various MOI (1–25) of AdRed. 48 hours later cells were harvested and 10 μg of total cell lysate was analyzed on 10% PAGE followed by immunoblot with rabbit anti-P450 reductase. (B) LLC-PK1 and L-MDR1 were stably transfected with P450-Reductase-Neo and lysates from individual clones analyzed on immunoblots with anti-P450 reductase IgG.

Ad3A4 at an MOI of either 25 or 50 addition of 1–10 MOI of AdRed maximally enhanced midazolam hydroxylation up to 5.8-fold over LLC-PK1/Ad3A4 cells or Caco-2/Ad3A4 (not shown). Based on these studies we chose for all subsequent analysis an Ad3A4 MOI of 50 and AdRed MOI of 5 or 10 for LLCPK1 and Caco-2 cells, respectively.

To directly compare the cell lines in this study with previous reports we determined marker CYP3A activities in cell *lysates* from the LLC-PK1 cells stably transfected with CYP3A4-Neo or CYP3A4-Hygro or LLC-PK1 cells transduced with Ad3A4. The rate of formation of 1′-hydroxymidazolam (1′R-OH-MDZ) was significantly higher in LLC-PK1 cells transduced at an Ad3A4 MOI of 100 compared to either of the stably transfected cells (Table 1). Activities were next compared in *microsomes* using a single saturating concentration (100 μM) of midazolam (Table 1). Consistent with CYP3A protein expression, LLC-Ad3A4/Red cells had significantly higher activity than Caco-Ad3A4/Red cells.

We expanded on the utility of the system by measuring metabolites released into the *medium* of cells transduced with Ad3A4 + AdRed and cultured on plastic dishes or in transwell culture. Cells incubated with 4 or 60 μM MDZ produced high amounts of 1′-OH-MDZ product (Table 2).

Testosterone 6β-hydroxylation assays were performed on microsomes prepared from LLC-PK1, L-MDR1, and Caco-2, as well as their counterparts transduced with either Ad3A4, Ad3A4 plus Adenovirus containing P450 reductase (AdRed) (Table 3). Activities were compared in microsomes using a single saturating concentration of testosterone (determined to be 100 μM). Microsomes isolated from parental cells showed no evidence of 6β testosterone hydroxylase activity. Cell lines transduced with CYP3A4 alone had significantly lower activity than cell lines transduced with both CY3A4 and oxidoreductase. The activities of LLC-PK1 Ad3A4/Red, L-MDR1 Ad3A4/Red, and Caco-2 Ad3A4/Red, were comparable to or even higher than that observed in mammalian expression systems such as

Table 1. Midazolam Hydroxylase Activity in Cell Lysates and Microsomes of LLC-PK1 and Caco-2 Cells Ectopically Overexpressing CYP3A4

Cells	1′-OH-MDZ pmol/min/mg	Fraction	Ref
LLC-PK1	Not detectable	Lysate	
LLC-3A4-Neo#2	2.52	Lysate	
LLC-3A4-MDR-Neo#9	2.11	Lysate	
LLC-3A4-Hygro#9**	3.13	Lysate	
LLC-3A4-Hygro#12**	2.11	Lysate	
LLC-Ad3A4 (MOI 100)	28.4	Lysate	
LLC-PK1/L-MDR1	5.3–11.2^	Microsomes	
LLC-Ad3A4/AdRed*	72.3-180^	Microsomes	
Caco-2	6.8	Microsomes	
Caco-2/Ad3A4/AdRed*	18.7	Microsomes	
Human liver	2.1–93.4 nmol/mg/hr	Microsomes	(30)
Human intestine	41.2 +/− 30.5 pmol/mg	Microsomes	(3)
Human intestine	9.2 pmol/mg	Homogenate	(3)

^ Range of activities among individual samples in different experiments.
* Ad3A4 (MOI = 50)/AdRed (MOI = 5).
** Ratio 1′ROH-MDZ/4-OH-MDZ = 3.0 and 2.77 for LLC-3A4-Hygro #12,9 respectively.

Table 2. Midazolam 1′-Hydroxylase Activity in LLC-PK1 and Caco-2 Cells Cultured on Plastic or in Transwell Culture Plate and Transduced with Ad3A4 Plus AdRed

Cells	35 mm Culture Wells	Substrate (μM)	Total pmoles 1′-OH-MDZ*
LLC-PK1	Plastic	60	0
LLC-Ad3A4/AdRed	Plastic	60	454
LLC-PK1	Transwell	4	ND
LLC-Ad3A4/AdRed	Transwell	4	246–262
LLC-PK1	Transwell	60	107.1
LLC-Ad3A4/AdRed	Transwell	60	153–379
L-MDR1	Plastic	60	0
L-MDR/Ad3A4/AdRed	Plastic	60	320
L-MDR1	Transwell	4	ND
L-MDR/Ad3A4/AdRed	Transwell	4	310–494
L-MDR1	Transwell	60	108.9
L-MDR1/Ad3A4/AdRed	Transwell	60	525–580
Caco-2	Plastic	60	27.8
Caco-2/Ad3A4/AdRed	Plastic	60	109
Caco-2	Transwell	4	ND
Caco-2/Ad3A4/AdRed	Transwell	4	63–101
Caco-2	Transwell	60	57
Caco-2/Ad3A4/AdRed	Transwell	60	71–321

Note: ND, Not done.
* Numbers represent the range of 1′-OH-MDZ product in the medium of all compartments (basal plus apical for transwell culture) following a 4 hr incubation with midazolam added to either the apical or basal compartment of cells transduced with 50 MOI Ad3A4 and 5 (LLC-PK1) or 10 (Caco-2) MOI AdRed.

Table 3. Testosterone 6β-Hydroxylase Activity in Cell Microsomes

Cell line (microsomes)	Testosterone 6β-hydroxylase activity @ 100 μM testosterone (pmol/mg/min)*	Km (μM)	Vmax (pmol/mg/min)
LLC-PK1	0	ND	ND
LLC-Ad3A4	182	14.8	196
LLC-Ad3A4/AdRed	956	33.0	1350
LLC-Ad3A4-P450-OR**	—	11.0	773
L-MDR1	0	ND	ND
L-MDR1-Ad3A4	188	30.0	259
L-MDR1-Ad3A4/AdRed	603	23.6	698
L-MDR1-Ad3A4-P450-OR**	—	15.9	311
Caco-2	0	ND	ND
Caco-2/Ad3A4	61.9	ND	ND
Caco-2/Ad3A4/AdRed	338	18.0	414
			~1250–8500***
Human liver microsomes	—	50–60 (20)	

Note: Caco-2 + CYP3A4 + Oxidoreductase = 4–5 pmol/mg/min (18). ND, Not detectable. Steady-state kinetic parameters were monitored by HPLC using isolated microsomal fractions, as described in the methods.
* Testosterone 6β-hydroxylase activity at 100 μM testosterone (saturating concentration).
** OR cell lines are stably transfected with NADPH-P450 reductase Neo.
*** 13-fold variation between human livers.

Hep G2 cells (19), V79 CHO cells (2), Caco-2 cells and lymphoblasts transduced with CYP3A4 alone or CYP3A4 plus NADPH P450Red (4,18). The catalytic testosterone 6β-hydroxylase activities were in the lower range of testosterone 6β-activity observed with a bank of human liver microsomes (20).

Next we determined the kinetic constants for testosterone 6β-hydroxylation in Caco-2, LLC-PK1 and L-MDR1 cells transduced by Ad3A4 (Table 3). K_m and V_{max} were 33.0 μM and 1350 pmol/mg/min, respectively, for LLC-PK1-Ad3A4/Red, 23.6 μM and 698 pmol/mg/min for L-MDR1-Ad3A4/Red, 18.0 μM and 414 pmol/mg/min for Caco-2 Ad3A4/Red (Table 3). These values are consistent with earlier studies and show the CYP3A4 is a low K_m enzyme for testosterone. The K_m values are slightly lower than those observed in insect cell microsomes (21) and human liver microsomes (20,21). The V_{max} values are in the range of human liver microsomes (20). In some experiments we observed differences between the Vmax values for LLC-PK1 and L-MDR1 cells (e.g., Table 3). This was likely attributable to the inherent difficulty in dissociating these cells and getting very precise cell counts and, ultimately, resulting in differences in Ad3A4 MOI/cell. More vigorous pipeting of the trypsinized cells was found to eliminate this variable cell counting and, thus, variable transduction efficiency between the LLC and L-MDR1 cells.

Next it was determined whether alterations in culture conditions might further increase the efficiency of AdV transduction. AdV infection requires expression of specific surface receptors. Integrins such as alphaV beta5 integrin participate in AdV adherence (22) and internalization (23). Because the expression of integrins in many cell types is markedly influenced by cell shape, cell density and cellular matrix (24,25), we determined whether alterations in any of these parameters might affect Ad3A4 transduction. We were particularly interested in the effects of the extracellular matrix, matrigel because it has also been shown to enhance expression of CYP3A in these cells (3). Caco-2 on plastic had no detectable CYP3A protein (Fig. 4), Ad3A4 transduction produced CYP3A4 in all Caco-2 cells cultured on either matrigel or plastic (Fig. 4). Despite a report that treatment of confluent Caco-2 with interleukin 1β can enhance adenovirus transduction (26), we observed no improvement of Ad3A4 expression in Caco-2 cells

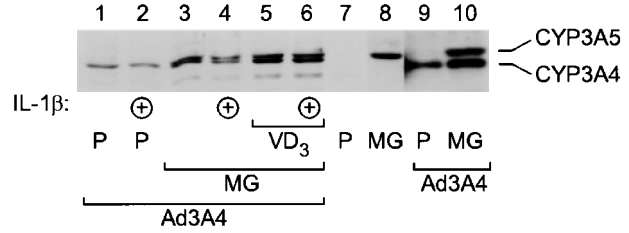

Fig. 4. Expression of CYP3A in Caco-2 cells cultured on various matrices. 35 mm plastic culture wells were coated with 350 μl liquid matrigel/well and matrigel allowed to solidify for 45 min–1 hr. Caco-2 cells were plated at 1×10^6 cells/well (with or without matrigel) on day 1. Some Caco-2 cells were treated with 2.5 μM 1,25-dihydroxyvitamin D3. On day 15 some cells were pretreated with recombinant interleukin-1β (5 ng/ml) (IL-1β) in serum free medium for 6 hrs and then cells were transduced with 50 MOI Ad3A4. 24 or 48 hrs later cells were harvested, matrigel dissolved by incubating cells in PBS with 5 mM EDTA on ice with frequent pipeting and cell pellets isolated, lysed in MSB and analyzed in immunoblots with anti-CYP3A IgG.

treated with this cytokine (Fig. 4). Higher CYP3A expression was observed in LLC-PK1 cells transduced at confluent versus sub-confluent density with Ad3A4 (not shown). Importantly, the confluent density and time course from plating until CYP3A4 analysis represents the optimal conditions used to culture LLC-PK1 and L-MDR1 cells for transwell transport experiments.

Because the ultimate goal was to analyze functional interactions between CYP3A and Pgp we determined whether transduction of adenovirus would affect membrane integrity. LLC-PK1 or Caco-2 cells transduced with AdVector or Ad3A4 showed similar transepithelial resistance (TEER) values. Next we assessed whether there was any change in Pgp function by measuring compartment-to-compartment movement of Pgp substrates across polarized cells transduced with Ad3A4 and AdRed in transwell culture. The rate of diffusion of vinblastine across LLC-PK1 cells in either direction (apical to basal and basal to apical) was unaffected by the presence of the AdV vector or Ad3A4 (not shown). Vinblastine transport in L-MDR1 cells (Fig. 5A) and digoxin transport across Caco-2 cells (Fig. 5B) were similar regardless of whether the cells were untreated or transduced with the AdVector or Ad3A4 (Fig. 5). These findings supports the feasibility of using Ad3A4 transduced LLC-PK1, L-MDR1 and Caco-2 cells for coupled transport/metabolism analysis.

DISCUSSION

Model cellular systems have previously been established to determine the way drugs interact singly with either CYP3A4 or Pgp. However, these drug metabolizing enzymes and transporters do not exist in isolation but are part of an integrated detoxification system. Thus, the challenges for the future are to generate model systems in which the dynamic interplay between drug transporting proteins and drug metabolizing enzymes can be explored. Towards that goal this is the first report on the functional analysis of LLC-PK1 and Caco-2 polarized cells expressing Pgp and transduced with Ad3A4 and AdRed. The results demonstrate that using the AdV approach yields robust expression of CYP3A4 activity and that Pgp function is unimpaired by introduction of the AdV. By simultaneously examining several methods for introduction and overexpression of CYP3A4 *in the same cellular background*, LLC-PK1, and side-by-side with Ad3A4 transduction of Caco-2, it was possible to directly compare each method and the two cell lines for expression of CYP3A protein and activities. AdV was clearly distinguished by its ability to produce the highest levels of CYP3A protein expression and associated catalytic activities followed by the episomal EBV vector which was superior to the neomycin vector in CYP3A4 production. Moreover, the testosterone 6β-hydroxylase activity of Ad3A4 transduced LLC-PK1 cells was superior to CYP3A4 expressed in many other extensively used cell model systems (2–4).

Many approaches are utilized to develop cell lines stably expressing CYPs (27), however, we are not aware of any report using adenovirus vectors. Transduction with recombinant adenovirus offers several advantages (reviewed in (1)). First, adenovirus infects most cell types. Second, the virus enters the cell but does not replicate. Third, adenovirus is well tolerated with post-infection viability 100% at low viral titers. Fourth, levels of the recombinant protein can represent as much as 15–20% of total cellular protein (1). Fifth, most AdV vectors

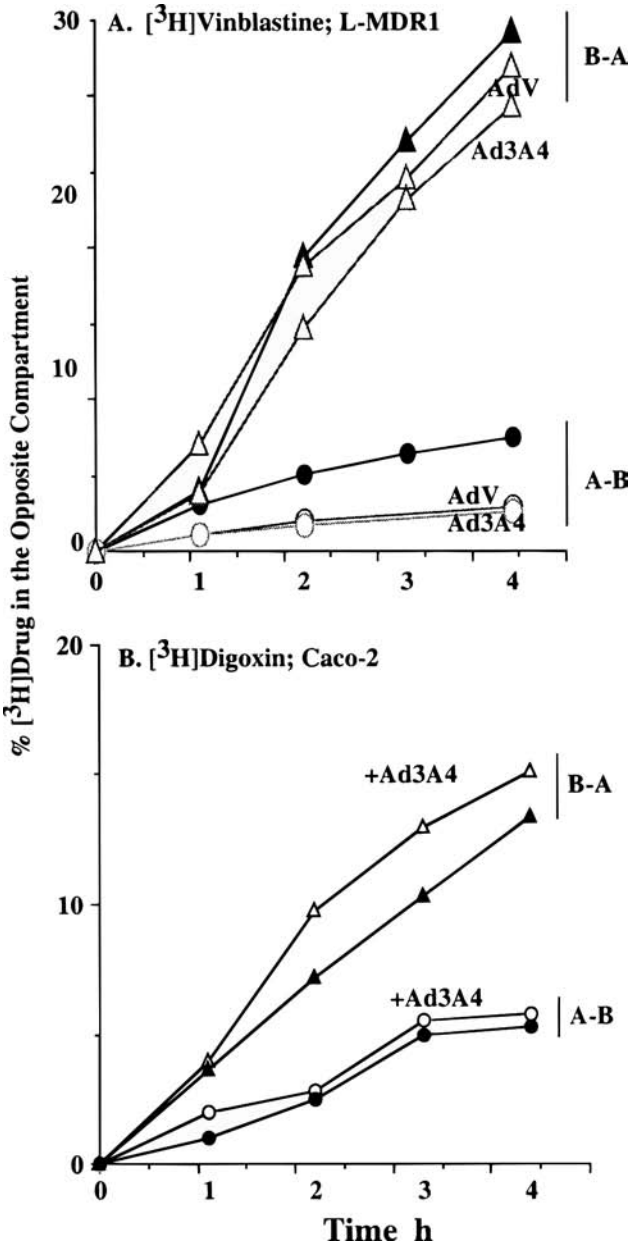

Fig. 5. Transepithelial transport of drugs across AdV transduced cells. (A) [^{3}H]vinblastine movement across L-MDR1 cells without (closed symbols) or with transduction of either AdVector (AdV) or Ad3A4 (both open symbols) and (B) [^{3}H]digoxin transport across Caco-2 cells without (closed symbol) or transduced with Ad3A4 (open symbols). Drug was added into either the apical (A) or basal (B) compartment and the % of radiolabel appearing in the opposite compartment determined at the indicated times.

can accommodate large cDNAs (about 8 kb), and the latest generation of so-called "gutless" AdV can accommodate up to 35 kb of foreign DNA. Sixth, recombinant AdV produced for *in vitro* studies can be used directly for generation of transgenic animals as the replication defective AdV can integrate into DNA of mouse eggs (28). Finally, as we have shown, multiple AdV can be simultaneously introduced into the same cell. Although AdV does not integrate stably, the viral episome is stable in cells, so when terminally differentiated cells (or confluent monolayers, such as those in transwell culture typically used for transport experiments) are infected, the viral genome will last essentially for the life of the cell.

A critical but as yet untested hypothesis in our systems is that because Pgp-mediated transport will influence the intracellular concentration of CYP3A substrate, Pgp could affect the extent of CYP3A-metabolites formed within the cell. To test this hypothesis we developed cellular systems to determine the pharmacokinetic interactions between the drug transporter and drug metabolizing enzyme. Thus, the ultimate goal of this study was to derive cells that polarize in culture, and have stable high levels of CYP3A4 in the presence or absence of the drug transporter Pgp. There are unique advantages and disadvantages of each model system chosen. Caco-2 cells are used extensively to screen for oral drug bioavailability, thus addition of CYP3A4, a protein known to be involved in first pass drug metabolism, enhances the utility of such a system. Caco-2 also maintain microvilli, making them most suitable for applicability as a surrogate for human intestinal mucosal epithelium. However, while these cells express Pgp they also express an unknown complement of other drug transporters that could complicate modeling the interactions between CYP3A4 and Pgp.

Although LLC-PK1 cells are not of human origin they offer several advantages over Caco-2. First, LLC-PK1 cells were superior to Caco-2 cells in AdV infectivity and expression of catalytically active CYP3A4. Second, L-MDR1 cells will readily allow us to measure Pgp transport kinetics of CYP3A4 substrates and metabolites in the absence of CYP3A4. By comparison, measuring the rates of formation of CYP3A metabolites in LLC-Ad3A4 and L-MDR-Ad3A4 cells will allow us to directly compare the rate and extent of CYP3A metabolism in the presence or absence of Pgp (ongoing experiments). Third, derivative LLC-PK1 cell lines expressing a variety of other transporters (e.g., MDR1, MRP1, SPGP, mdr1a, mdr1b, MRP3, etc) currently exist (10,11,29). Therefore, the interactions of other Phase I and Phase II drug metabolizing enzymes with the drug transporters can be explored in this model cell system. We envision cell systems, such as those developed and characterized in this report containing CYP3A4 with and without Pgp, as the paradigm for such studies.

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