[33] Functional Expression of Human P-Glycoprotein from Plasmids using Vaccinia Virus–Bacteriophage T7 RNA Polymerase System

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Introduction

Transient expression of recombinant proteins in mammalian cells provides a rapid and convenient way to study the biochemistry of a protein of interest without the confounding effects that are normally associated with many of the stable expression systems.[1] However, many of the systems currently available do not allow for high-level expression of protein. A recent protocol designed to circumvent this problem uses HEK293 cells and histidine-tag affinity chromatography to purify partially and enrich for transiently expressed protein for use in *in vitro* biochemical analyses.[2,3] Transient viral transductants are known to produce larger amounts of recombinant protein within 24–48 hr; however, constructing the recombinant viruses is time consuming. A notable exception is the recombinant vaccinia-T7 RNA polymerase (vaccinia-T7) system developed by Bernard Moss and colleagues at the National Institutes of Health.[4–6] In this system, on infection with a recombinant vaccinia virus encoding the bacteriophage T7 RNA polymerase (vTF7-3), a cotransfected cDNA under control of the bacteriophage T7 promoter on a plasmid is expressed at high levels.[4,7]

For transient expression using the vaccinia-T7 system, virtually any mammalian cell can serve as the host and typically 70–80% of cells will express the protein of interest. Because it is believed that viral entry of the vaccinia virus apparently involves fusion of the viral envelope with the plasma membrane, this high transfection efficiency in the vaccinia-T7 system can be attributed to the fact that the cationic liposome-mediated transfec-

[1] C. A. Hrycyna, S. Zhang, M. Ramachandra, B. Ni, I. Pastan, and M. Gottesman, *in* "Multidrug Resistance in Cancer Cells: Cellular, Biochemical, Molecular, and Biological Aspects" (S. Gupta and T. Tsuruo, eds.), p. 29. John Wiley and Sons, New York, 1996.
[2] T. W. Loo and D. M. Clarke, *J. Biol. Chem.* **270,** 21449 (1995).
[3] T. W. Loo and D. M. Clarke, *Methods Enzymol.* **292,** [35], 1998 (this volume).
[4] T. R. Fuerst, E. G. Niles, F. W. Studier, and B. Moss, *Proc. Natl. Acad. Sci. U.S.A.* **83,** 8122 (1986).
[5] B. Moss, *Proc. Natl. Acad. Sci. U.S.A.* **93,** 11341 (1996).
[6] B. Moss, *Science* **252,** 1662 (1991).
[7] T. R. Fuerst, P. L. Earl, and B. Moss, *Mol. Cell Biol.* **7,** 2538 (1987).

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tion and infection steps are performed simultaneously allowing coentry of plasmid DNA and virus to each cell.[6] This procedure, however, is not completely efficient; although all of the cells are infected by the virus, only 70–80% contain plasmid DNA. Transfected plasmids are transcribed in the cytoplasm of vaccinia virus-infected cells, avoiding the requirement of plasmid transit to the nucleus, thus allowing virtually all transfected cells to express the protein of interest. Additionally, the presence of an encephalomyocarditis virus internal ribosomal entry site (IRES) sequence downstream from the T7 promoter on the expression plasmid makes the translation of the viral transcripts cap independent, significantly enhancing the expression level.[8] Alternatively, recombinant viruses encoding a similar expression cassette (T7 promoter-IRES-cDNA-T7 terminator) can also be generated.[9]

We have successfully expressed human P-glycoprotein (Pgp) in a variety of mammalian cells using this recombinant vaccinia-T7 RNA polymerase system and determined it to be a useful tool for rapidly analyzing the functional properties of Pgp.[10] The coinfection and transfection expression system is well suited for studying great numbers of mutant/chimeric constructs since large amounts of recombinant Pgp can be expressed rapidly and the cells remain intact allowing for whole-cell *in vivo* experiments to be performed (Fig. 1). Importantly, it is unnecessary to impose any drug selection on these cells, eliminating bias due to any possible pleiotropic cellular effects of drug exposure. We have also generated recombinant vaccinia viruses encoding Pgp[9]; however, although this method somewhat increases the amount of protein made and allows for virtually 100% of infected cells to express Pgp, this pursuit is time consuming and is not amenable to the study of numerous constructs. For mutational studies, there is no need to generate a new virus for each construct, because almost all functional aspects of Pgp can be studied upon protein expression using the infection–transfection procedure.

The protocols outlined in this article describe various methods used to express and functionally characterize human Pgp including the infection–transfection procedure, immunoblot analysis, photoaffinity labeling with nucleotide and substrate analogs, drug-stimulatable ATPase activity assays, fluorescent and radioactive substrate accumulation/efflux assays in intact cells, and determination of cell surface localization by flow cytometry. We

[8] O. Elroy-Stein, T. R. Fuerst, and B. Moss, *Proc. Natl. Acad. Sci. U.S.A.* **86,** 6126 (1989).
[9] M. Ramachandra, M. M. Gottesman, and I. Pastan, *Methods Enzymol.* **292,** [32], 1998 (this volume).
[10] M. Ramachandra, S. V. Ambudkar, M. M. Gottesman, I. Pastan, and C. A. Hrycyna, *Mol. Biol. Cell* **7,** 1485 (1996).

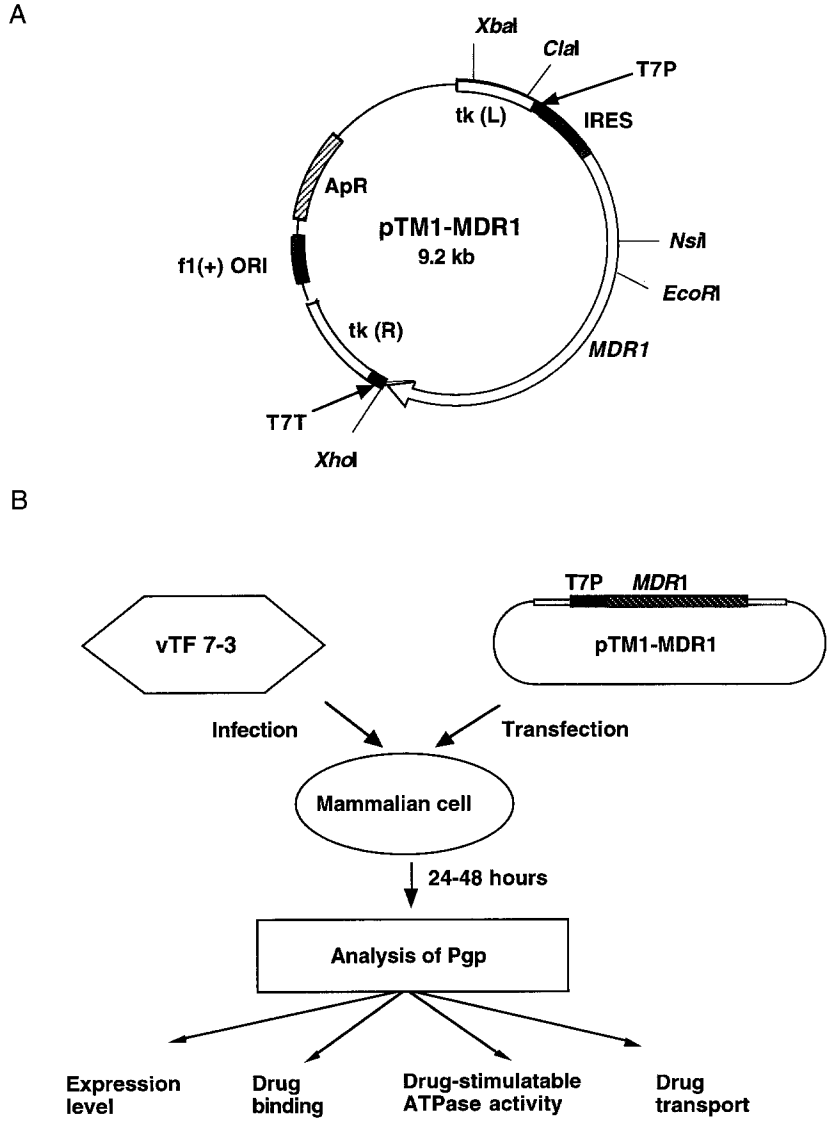

FIG. 1. Pgp-expression plasmid and schematic diagram of the infection–transfection protocol and functional assays. (A) The unique restriction sites and main features of the expression plasmid pTM1-*MDR1* are shown. In this vector, *MDR1* is controlled by the T7-promoter (T7P) and the presence of an IRES sequence between the T7-promoter and *MDR1* sequence facilitates cap-independent translation on transfection of cells infected with vTF 7-3, a recombinant vaccinia virus encoding T7 RNA polymerase. The T7 terminator (T7T) is present at the 3′-end of the *MDR1* cDNA. The expression cassette is flanked by segments of the vaccinia

have successfully adapted these assays for use with virally infected–transfected cells.[10]

Techniques and Protocols

Construction of Expression Vectors

The parental plasmid vector pTM1[11] is most commonly used to drive expression of cDNAs in this recombinant vaccinia virus-bacteriophage T7 RNA polymerase system. This plasmid, propagated in *Escherichia coli,* contains the T7 promoter, an IRES, a multiple cloning site for insertion of the cDNA of interest, and the T7 terminator (Fig. 1). It is ideal to place the cDNA of interest at the *Nco*I site present at the 3′-end of the IRES to maximize transcription efficiency. The 3′-end of the cDNA can be cloned into any site preceding the T7 terminator sequence. We used the following protocol to clone the human *MDR1* cDNA into the transfer vector pTM1 to obtain pTM1-*MDR1.*

Materials

Restriction enzymes (New England Biolabs, Beverly, MA)
Calf intestinal phosphatase (CIP) and polynucleotide kinase (New England Biolabs)
Competent DH5α *E. coli* (Life Technologies, Grand Island, NY)

Method

1. Obtain the DNA fragment (1183 bp) between the second codon and the *Eco*RI site in the coding sequence from human *MDR1* by polymerase chain reaction (PCR) using pMDRXS(WT) (Sugimoto, Gottesman, and Pastan, unpublished data) as a template.
2. Phosphorylate the 5′-end with polynucleotide kinase and digest with *Eco*RI and subclone the PCR fragment into the linearized vector

[11] O. Elroy-Stein and B. Moss, *in* "Current Protocols in Molecular Biology" (F. M. Ausubel, R. Brent, R. E. Kinston, D. D. Moore, J. A. Smith, J. C. Seidman, and K. Struhl, eds.), p. 16.19.1–16.19.9. John Wiley and Sons, New York, 1991.

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virus thymidine kinase (tk) gene, which allows for the use of this construct to generate and isolate recombinant vaccinia virus. (B) Outline of the infection–transfection protocol and functional assays that can be performed in the infected–transfected cells. [Reproduced from *Molecular Biology of the Cell* (1996, Volume 7, 1485–1498), with permission of the American Society for Cell Biology.]

pTM1[11] that was prepared by digestion with *Nco*I, treatment with the Klenow fragment of *E. coli* DNA polymerase followed by digestion with *Eco*RI, to obtain pTM1-*MDR1*(NE).

3. Double-digest pTM1-*MDR1*(NE) with *Nsi*I and *Xho*I and ligate to the remaining coding sequence of *MDR1* isolated from pMDRXS(WT) by digesting with the same enzymes. The resultant expression vector is pTM1-*MDR1*.
4. Mutations can be readily introduced into pTM1-*MDR1* by cassette replacement of unique fragments made by sequence overlap PCR techniques.[12] All sequences should be subsequently verified by either manual or automated DNA sequencing (PRISM™ Ready Reaction DyeDeoxy™ Terminator Sequencing Kit, Perkin-Elmer Corporation, Norwalk, CT).

Expression of Pgp by an Infection–Transfection Protocol

The protocol[11] described next has been optimized for the amount of Pgp expression in human osteosarcoma and HeLa cells. These cell lines were chosen because they have negligible amounts of endogenous Pgp expression and low basal membrane-associated ATPase activity, and they are easily transfected. Similar conditions can also be used for other cell types although varying the time of expression as well as the temperature of incubation may be necessary. Cells are normally harvested at 24 hr for assays with intact cells and at 48 hr for isolating crude membranes after infection–transfection.

Materials

Minimum essential medium with Earle's salts (Life Technologies) supplemented with 4.5 g/liter glucose, 5 m*M* L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, and 10% fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT) (EMEM). HeLa cells are grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 g/liter glucose, 5 m*M* L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, and 10% FBS (Quality Biological, Gaithersburg, MD)

Human osteosarcoma (HOS) American Type Culture Collection (ATCC) cells or HeLa cells were propagated as monolayer cultures at 37° in 5% CO_2 in their respective medium

[12] R. Higuchi, *in* "PCR Technology" (H. A. Erlich, ed.), pp. 61–70. W. H. Freeman and Company, New York, 1992.

Recombinant vaccinia virus encoding bacteriophage T7 RNA polymerase (vTF7-3) American Type Culture Collection (ATCC), with vTF7-3 propagated and purified as previously described[9-11,13]
Lipofectin® reagent (1 mg/mL) (Life Technologies)
Opti-MEM reduced serum medium (Life Technologies)
pTM1-*MDR1* plasmid DNA

Method

1. Split the cells the day before into 75-cm^2 tissue culture flasks such that they will be about 70–80% confluent on the day of infection–transfection.
2. For each transfection, place 3.0 mL Opti-MEM into a 15-mL polystyrene tube. Since DNA–Lipofectin complexes are known to bind to polypropylene, it is essential to use polystyrene tubes.
3. Vortex the Lipofectin solution, add 45 μl (45 μg) Lipofectin reagent to the Opti-MEM and vortex.
4. Add DNA to attain a 3:1 lipid:DNA ratio by adding 15 μg DNA (pTM1 as control or desired pTM1-*MDR1* construct) to the medium containing Lipofectin and gently mix by swirling. Do not vortex the solution after adding the DNA.
5. Incubate the tubes at room temperature for 20–30 min.
6. Five minutes before the end of the incubation period, add vTF7-3 to Opti-MEM and vortex 15–30 sec. Routinely, use 10 plaque forming units (pfu)/cell and use 0.5 mL of Opti-MEM per flask. If the virus is to be used for more than one flask, a master mix should be prepared. However, first vortex the virus in a small volume of Opti-MEM (0.5–3 mL), then dilute to the appropriate volume and vortex again to mix. If a nonpurified virus is being used, sonicate the virus in 0.5–3 mL Opti-MEM for 30 sec in a bath sonicator filled with 50% ice in water, dilute accordingly, and then vortex to mix.
7. Wash the cells once with 5–10 mL Opti-MEM medium. Add the 0.5 mL of the viral dilution to the flask and then gently place the DNA–Lipofectin mix directly on the cells. Mix the solutions well in the flask and incubate for 4 hr at 32° or 37°. For the first hour, gently swirl the solution in the flask every 15 min. The total volume of the infection–transfection is 3.5 mL.

[13] P. E. Earl, N. Cooper, and B. Moss, *in* "Current Protocols in Molecular Biology" (F. M. Ausubel, R. Brent, R. E. Kinston, D. D. Moore, J. A. Smith, J. C. Seidman, and K. Struhl, eds.), pp. 16.16.1–16.16.7. John Wiley and Sons, New York, 1991.

8. After 4 hr, add 12 ml complete medium and incubate for desired periods of time. Expression can be seen as early as 8 hr post–infection–transfection and maximum expression is usually achieved by 48 hr. Functional studies in intact cells are carried out at 20–24 hr post–infection–transfection.

Preparation of Crude Membranes

Crude membrane preparations from infected–transfected cells can be used for a number of applications including immunoblot analysis, photoaffinity nucleotide- and drug-binding studies, and drug-stimulatable ATPase activity assays.

Materials

Phosphate buffered saline without Ca^{2+} and Mg^{2+} (PBS)

Hypotonic lysis buffer: 10 m*M* Tris (pH 7.5), 10 m*M* NaCl, and 1 m*M* $MgCl_2$. Add the following reagents just prior to use: 1 m*M* dithiothreitol (DTT), 1% (v/v) aprotinin solution (0.1 U/mL) (Sigma, St. Louis, MO), and 2 m*M* 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) (ICN, Irvine, CA)

Micrococcal nuclease (*Staphylococccus aureus*) (Pharmacia Biotech, Piscataway, NJ)

Dounce homogenizer with pestle A

TSNa resuspension buffer: 10 m*M* Tris (pH 7.5), 50 m*M* NaCl, 250 m*M* sucrose. Add 1 m*M* DTT, 1% (v/v) aprotinin solution (0.1 U/mL), and 2 m*M* AEBSF just prior to use

Method

1. Harvest the cells by scraping and wash twice in PBS containing 1% (v/v) aprotinin solution (0.1 U/mL).
2. Resuspend the cell pellet in hypotonic lysis buffer in a volume of approximately 0.5 mL per 75-cm^2 tissue culture flask and freeze at −80°. Subsequently, thaw the cells and incubate on ice for 30–45 min.
3. Disrupt the cells using 50 strokes with a Dounce homogenizer using tight fitting pestle A only.
4. Dilute the homogenate two-fold with hypotonic lysis buffer and subsequently remove the undisrupted cells and nuclear debris by centrifugation at 500*g* for 10 min at 4°.
5. Transfer the low-speed supernatant to a new tube and incubate with micrococcal nuclease (50 U/mL) in the presence of 1 m*M* $CaCl_2$ for 20–30 min on ice.

6. Collect the membranes by centrifugation for 60 min at 100,000*g*, and resuspend in resuspension buffer containing 10% glycerol using a 1–3 ml syringe and a blunt-ended 23-gauge needle.
7. Store the resuspended membranes in small aliquots at −70° immediately and determine the protein content by a modified Lowry method[14] using bovine serum albumin as a standard.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE) and Immunoblot Analysis

Materials

SDS–PAGE gels
Laemmli SDS–PAGE sample loading buffer[15]
Running buffer: 25 m*M* Tris-glycine (pH 8.3) and 0.2% (w/v) SDS
Transfer buffer: 25 m*M* Tris-glycine (pH 8.3) and 0.2% (w/v) SDS + 20% (v/v) methanol (SDS optional)
Phosphate buffered saline without Ca^{2+} and Mg^{2+} with 0.05% (v/v) Tween 20 (PBST)
Nitrocellulose membranes (0.45-μm pore size, Schleicher and Schuell, Keene, NH)
Monoclonal anti-P-glycoprotein antibody C219 (Centocor, Malvern, PA)[16]
Secondary antibody: peroxidase-conjugated goat anti-mouse IgG (H + L) (Life Technologies)
Nonfat dry milk

Method

1. Incubate membrane preparations in an appropriate volume of Laemmli SDS–PAGE sample loading buffer (1× final concentration) at room temperature for 30 min.
2. Perform electrophoresis on 8.0% polyacrylamide gels at 170 V in running buffer.
3. Electroblot the proteins onto a nitrocellulose membrane in transfer buffer at 400 mA (constant amperage) for 60 min in the presence of an ice block.
4. After transfer, block the membrane for 30 min at room temperature in PSBT containing 20% (w/v) nonfat dry milk.

[14] J. L. Bailey, *in* "Techniques in Protein Chemistry," p. 340. Elsevier Publishing, New York, 1967.
[15] U. K. Laemmli, *Nature* **227,** 680 (1970).
[16] E. Georges, G. Bradley, J. Gariepy, and V. Ling, *Proc. Natl. Acad. Sci. U.S.A.* **87,** 152 (1990).

5. Incubate the blot in a 1:2000 dilution of C219 monoclonal antibody prepared in PBST containing 5% (w/v) nonfat dry milk for 2 hr to overnight at room temperature.
6. Wash the blot three times for 15 min in PSBT.
7. Incubate the blot for 60 min at a 1:4000 dilution of secondary antibody in PBST containing 5% (w/v) nonfat dry milk.
8. Wash three times for 15 min in PBST.
9. Develop the blot using an enhanced chemiluminescence kit (Amersham Life Science, Arlington Heights, IL) as per manufacturer's instructions.

[α-^{32}P]8-Azidoadenosine-5'-triphosphate Labeling of Pgp and Immunoprecipitation

Materials

[α-^{32}P]8-Azidoadenosine-5'-triphosphate ([α-^{32}P]8-azido-ATP) (specific activity 23 Ci/mmol; 2.0 mCi/mL) (ICN)

TSNa resuspension buffer: 10 m*M* Tris (pH 7.5), 50 m*M* NaCl, 250 m*M* sucrose. Add 1 m*M* DTT 1% (v/v) aprotinin solution (0.1 U/mL) (Sigma), and 2 m*M* AEBSF (ICN) just prior to use

2× Labeling buffer: 100 m*M* Tris-HCl (pH 7.5) with 2 m*M* DTT, 2% (v/v) aprotinin solution (0.2 U/mL) (Sigma), and 4 m*M* AEBSF (ICN) added just prior to use

1 *M* $MgCl_2$

1× RIPA buffer: 20 m*M* Tris-HCl (pH 7.2), 150 m*M* NaCl, 1% (v/v) Triton-X 100, 1% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate (SDS) 1 m*M* ethylenediaminetetraacetic acid (EDTA)

Protein A agarose: 45–55% suspension in 20 m*M* sodium phosphate (pH 7.2), 0.15 *M* NaCl, 0.02% (w/v) merthiolate (Life Technologies)

Method

1. Dilute the membrane protein (50–100 μg total protein) in TSNa resuspension buffer or 1× labeling buffer containing 10 m*M* $MgCl_2$ (final concentration) to a final volume of 97.5 μl in a 1.5-ml microcentrifuge tube.
2. Incubate on ice for 3 min.
3. Add 5 μCi [α-^{32}P]8-azido-ATP per tube under subdued light conditions and incubate in the dark for 10 min on ice.
4. UV cross-link by exposing the reaction mix to a UV lamp at 365 nm (Black-Ray lamp, model XX-15, UVP, Upland, CA) for 15 min on ice.

5. Analyze the cross-linked products by immunoprecipitation as described in the following steps.
6. To each sample add 600 μL 1× cold RIPA buffer and antibody (e.g., 8 μL of anti-Pgp polyclonal antibody 4007.[17])
7. Incubate at 4° for 1 hr on a rotary shaker.
8. Add 25 μL protein A agarose beads and continue to incubate at 4° for an additional 2 hr on a rotary shaker.
9. Wash the beads three times with 1 mL 1× cold RIPA buffer. Pulse down beads at 14,000*g* for 10 sec, remove supernatant, and repeat twice.
10. Elute protein by incubation with 30 μL 2× SDS–PAGE sample buffer at room temperature for 30–60 min.
11. Load sample, including beads, and perform SDS–PAGE, fix the gel in a solution containing 30% (v/v) methanol and 10% (v/v) acetic acid for 30 min and subsequently dry the gel. Alternatively, transfer the proteins from the gel to a nitrocellulose membrane and subject the membrane to autoradiography.

Photoaffinity Labeling of Pgp with [^{3}H]Azidopine or [^{125}I]Iodoarylazidoprazosin

Materials

Iscove's modified Dulbecco's medium supplemented with 5% FBS or calf serum (CS) (IMEM) (Life Technologies)

Trypsin-EDTA: 0.25% (w/v) trypsin, 1 m*M* EDTA

Phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS) with 1% (v/v) aprotinin solution (0.1 U/mL) (Sigma)

TD buffer: 10 m*M* Tris (pH 8.0), 0.1% (v/v) Triton X-100, 10 m*M* $MgSO_4$, 2 m*M* $CaCl_2$. Add 1% (v/v) aprotinin solution (0.1 U/mL) (Sigma) 2 m*M* AEBSF (ICN), 1 m*M* DTT, and 20 μg/ml deoxyribonuclease I (DNase) (Sigma) just prior to use. Alternatively, 50 U/mL micrococcal nuclease (*S. aureus*) (Pharmacia Biotech) in the presence of 1 m*M* $CaCl_2$ can be used in place of DNase

2× Labeling buffer: 100 m*M* Tris-HCl (pH 7.5) with 2 m*M* DTT, 2% (v/v) aprotinin solution (0.2 U/mL) (Sigma), and 4 m*M* AEBSF (ICN) added just prior to use

5× Laemmli SDS–PAGE sample loading buffer[15]

ENLIGHTNING® (DuPont-NEN, Boston, MA)

[17] S. Tanaka, S. J. Currier, E. P. Bruggemann, K. Ueda, U. A. Germann, I. Pastan, and M. M. Gottesman, *Biochem. Biophys. Res. Commun.* **166,** 180 (1990).

[^{3}H]Azidopine (specific activity, 46 Ci/mmol) (Amersham Life Science)
[^{125}I]Iodoarylazidoprazosin ([^{125}I]IAAP) (specific activity, 2200 Ci/mmol) (DuPont-NEN)

Labeling of Pgp in Intact Cells

Method

1. Harvest cells by trypsinization 24 hr after infection–transfection and wash once with 15 mL of IMEM.
2. Resuspend pellet in 1 mL IMEM, count cells, and aliquot 1×10^6 cells into a 1.5-mL microcentrifuge tube.
3. Centrifuge the cells for 30 sec at 14,000*g*.
4. Remove the supernatant and resuspend the cells in 98 μL PBS containing 2 m*M* AEBSF (ICN) added just prior to use.
5. Add 1 μCi of [^{3}H]azidopine or [^{125}I]IAAP (1–2 μL). For binding competition assays, 1 μL of 100× concentrated drugs dissolved in dimethyl sulfoxide (DMSO) are first added as the competitors (1% final concentration of DMSO) and incubated for 3–5 min at room temperature before adding the labeled drug.
6. Incubate the cells at room temperature on a rotary shaker in the dark for 60 min.
7. After incubation, directly expose the samples to a 365-nm UV lamp (Black-Ray lamp, UVP) on ice for 30 min.
8. After UV cross-linking, add 500 μL of cold PSB and centrifuge the cells for 30 sec at 14,000*g*
9. Resuspend the pellets in 100 μL TD buffer (1×10^6/100 μL TD buffer).
10. Subject the cells to three freeze–thaw cycles to lyse the cells. Freeze on dry ice for 5 min and thaw at 37° for 2–3 min each cycle and vortex between cycles.
11. Add 25 μL 5× Laemmli sample buffer to the resulting lysate and perform SDS–PAGE on an appropriate aliquot (50–60 μL). Load 5–10 μL of the sample on an SDS–PAGE gel run in parallel to compare sample loading by immunoblot analysis as described earlier.
12. Alternatively, the lysate can be subjected to immunoprecipitation as described earlier.
13. Fix gels in a solution containing 30% (v/v) methanol and 10% (v/v) acetic acid for 30 min.
14. Treat gels containing ^{3}H-labeled samples with ENLIGHTNING for 20 min, dry, and subject to autoradiography.
15. Immediately dry gels containing the ^{125}I-labeled samples without treatment with ENLIGHTNING and subject to autoradiography.

Labeling of Pgp in Membrane Preparations

Method

1. Dilute membrane preparations (25–50 μg) to 98 μL in 1× labeling buffer.
2. Add 1 μCi of [^{3}H]azidopine or [^{125}I]IAAP (1–2 μL). For binding competition assays, first add 1 μL of 100× concentrated drugs dissolved in DMSO (1% final concentration of DMSO to the membrane preparations and incubate at room temperature for 3 min. Subsequent to this incubation, add the [^{3}H]azidopine or [^{125}I]IAAP solution.
3. Incubate the sample at room temperature in the dark for 10 min.
4. After incubation, directly expose the samples to a 365-nm UV lamp (Black-Ray lamp, UVP) on ice for 15 min.
5. After UV cross-linking, add an appropriate volume of SDS–PAGE sample buffer (1× final concentration) and perform SDS–PAGE as described earlier. At this point, alternatively, the sample can be subjected to immunoprecipitation as described earlier.
6. Treat gels as described earlier.

Measurement of ATPase Activity

Pgp-associated substrate-stimulated ATPase activity is measured by determining the vanadate-sensitive release of inorganic phosphate from ATP with a colorimetric method as previously described[18] with some modifications. The vanadate-sensitive activities in the presence and absence of substrate are calculated as the differences between the ATPase activities obtained in the presence and absence of 300 μM sodium orthovanadate. Briefly, membrane suspensions are first incubated at 37° for 5 min in the reaction mixture assay buffer and then substrates are added from stock solutions prepared in DMSO and the assay mixtures are then incubated for 3 min at 37°. The final concentration of DMSO in the assay medium is 1% (v/v), a concentration that does not exhibit any effect on the ATPase activity. The reactions are subsequently started by the addition of 5 mM ATP to the assay mixtures and incubated at 37° for desired periods of time (usually 20 min). Reactions are stopped by the addition of 5% (w/v) SDS solution and the amount of inorganic phosphate (P_i) released is measured by a colorimetric reaction.

[18] B. Sarkadi, E. M. Price, R. C. Boucher, U. A. Germann, and G. A. Scarborough, *J. Biol. Chem.* **267,** 4854 (1992).

Materials

Phosphate (P_i) reagents: 1% ammonium molybdate (or molybdic acid, ammonium salt) in 2.5 *N* sulfuric acid and 0.014% antimony potassium tartarate). Add 50 ml distilled water and 6.9 ml concentrated sulfuric acid (36.2 *N* solution) to a 250-ml glass beaker and then add 1 g ammonium molybdate powder and 14 mg of antimony potassium tartrate and stir for 20 min in a hood. Make up the final volume to 100 ml with distilled water and store the solution in a glass bottle covered with aluminum foil at room temperature

2× assay buffer (make fresh): 100 m*M* Tris (pH 7.5), 10 m*M* sodium azide, 4 m*M* ethylene-bis(oxyethylenenitrilo)tetraacetic acid (EGTA) (pH 7.0), 2 m*M* ouabain, 4 m*M* DTT, 100 m*M* KCl, and 20 m*M* $MgCl_2$

5% (w/v) SDS

1% (w/v) ascorbic acid prepared fresh and stored on ice

10 m*M* sodium orthovanadate: Prepare a fresh stock of 30–40 m*M* and incubate solution at 100° for 3 min prior to use. Read OD_{268} and adjust to a concentration of 10 m*M* (OD 3.6 = 1 m*M*)

100× Concentrated stocks of various drugs to be tested prepared in DMSO

100 m*M* Adenosine 5′-triphosphate (ATP) (disodium salt), pH 7.0. Store in aliquots at −80°

1 m*M* potassium phosphate. Store in aliquots at −20°

Method

1. Carry out the reaction in duplicate in either 13 × 100 mm or 12 × 75 mm glass tubes and use 10–20 μg membrane protein/reaction.
2. Mix the components as shown in Table I. Note that the volumes given are in microliters.
3. Incubate the components mixed as described in Table I at 37° for 3 min.
4. Add 5 μL 100 m*M* ATP.
5. Incubate at 37° for the desired time, add 100 μl 5% (w/v) SDS, and vortex.
6. To develop the color add 400 μl P_i reagent, vortex, and add 500 μl water. Add 200 μl 1% (w/v) ascorbic acid to each tube and vortex immediately. Incubate at room temperature for 10 min.
7. Read the optical density at 880 nm using ATP alone in assay buffer (tube V in Table I) as the blank.
8. Prepare the phosphate standard curve by taking 0, 10, 20, 30, 40, 50, 75, and 100 μl of 1 m*M* phosphate standard in a final volume of 100

TABLE I
SEQUENTIAL ADDITIONS[a] TO MEASURE SUBSTRATE-STIMULATED ATPASE ACTIVITY

Tube	I	II	III	IV	V
2× assay buffer	50	50	50	50	50
H_2O	(Adjust the final volume to 100 μl)				
10 m*M* sodium orthovanadate	—	3	—	3	—
Membranes	10–20 μg/tube				—
Incubate at 37° for 3 min and then add the following:					
Dimethyl sulfoxide (DMSO)	1	1	—	—	1
100× substrate stock	—	—	1	1	—

[a] Volumes are given in microliters.

μl and add 100 μl 5% (w/v) SDS, 400 μl P_i reagent, 500 μl water, and 200 μl 1% (w/v) ascorbic acid. Incubate the samples at room temperature for 10 min and read the optical density at 880 nm. The color is stable for 30 min. Usually, 1 OD corresponds to 58–62 nmol of phosphate.

Determination of Cell-Surface Expression of Pgp by MRK-16 Staining

Materials

Trypsin-EDTA: 0.25% (w/v) trypsin, 1 m*M* EDTA
Purified mouse IgG_{2a}, kappa (anti-TNP) (PharMingen, San Diego, CA)
MRK-16 monoclonal antibody[19] that recognizes an external epitope of human Pgp (1 mg/mL) (Hoechst, Japan)
FITC-labeled anti-mouse IgG_{2a} (PharMingen)
Iscove's modified Dulbecco's medium supplemented with 5% (v/v) FBS or CS (IMEM) (Life Technologies)
Phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS)
Fluorescence-activated cell sort flow cytometer (Becton-Dickinson, San Jose, CA)
6-mL 12 × 75-mm polystyrene round-bottom tubes with caps (Falcon, Becton-Dickinson, Lincoln Park, NJ)

Method

1. Remove cells from flasks 24 hr after infection–transfection by trypsinization; wash once in IMEM.

[19] H. Hamada and T. Tsuruo, *Proc. Natl. Acad. Sci. U.S.A.* **83,** 7785 (1986).

2. Incubate 3.5–5 $\times$ 10^5 cells in a final volume of 200 μL of the same medium with 6 μg of MRK-16 or purified mouse IgG_{2a} (as an isotype control) in 6 mL polystyrene tubes with caps.
3. Incubate at 4° for 20–30 min.
4. Dilute cell suspensions to 4.5 ml in IMEM and centrifuge at 200*g* for 5 min.
5. Aspirate the wash medium and resuspend cell pellet in 200 μL IMEM containing 3 μg FITC-labeled anti-mouse IgG.
6. Incubate at 4° for 20–30 min under subdued light conditions.
7. Dilute cell suspensions to 4.5 ml in IMEM, centrifuge at 200*g* for 5 min.
8. Remove the medium and resuspend the cells in 500 μl of IMEM and centrifuge again at 200*g* for 5 min.
9. Remove the wash medium and resuspend the cell pellet in 350–500 μl PBS and analyze by fluorescence-activated cell sort flow cytometry (FACSort) (Becton-Dickinson FACS system)

Fluorescent Substrate Efflux and Accumulation Assays in Intact Cells

Materials

Phosphate-buffered saline without Ca^{2+} and Mg^{2+}
Trypsin-EDTA: 0.25% w/v trypsin, 1 m*M* EDTA
Iscove's modified Dulbecco's medium supplemented with 5% (v/v) FBS or CS (IMEM) (Life Technologies)
DMEM without glucose and phenol red (glucose-free DMEM) (Life Technologies)
Glucose-containing DMEM (Bio-Whittaker, Walkersville, MD)
2-Deoxy-glucose (Sigma)
Sodium azide (Sigma)
Rhodamine-123 (Eastman Kodak Co., Rochester, NY)
6-mL 12 $\times$ 75-mm polystyrene round-bottom tubes with caps (Falcon)
FACSort (Becton-Dickinson)

ATP-Dependent Rhodamine-123 Efflux Assay in Intact Cells

Method

1. Harvest and wash cells as described earlier for MRK-16 antibody staining.
2. To determine energy-dependent rhodamine-123 efflux, incubate 500,000 cells in either glucose-free DMEM containing 5 m*M* 2-deoxy-glucose and 10 m*M* sodium azide (to deplete energy) or 25 m*M*

glucose-containing DMEM for 20 min at room temperature in 6-mL polystyrene tubes with caps.
3. Pellet cells by centrifugation at 200*g* for 5 min and add 4.5 mL of their respective medium containing 0.5 μg/ml rhodamine 123 added from a stock of 1 mg/ml in ethanol.
4. Incubate at 37° for 40 min and pellet the cells by centrifugation at 200*g*.
5. Remove medium, resuspend cells in 0.5 mL of their respective media without rhodamine-123, and add an additional 4 mL of the same media.
6. Incubate for an additional 40 min at 37°.
7. Pellet cells and resuspend in 450 μl of ice-cold PBS and immediately analyze by FACS.

Fluorescent Substrate Accumulation Assay

Method

1. For fluorescent drug accumulation measurements, incubate 500,000 cells in 5 ml of IMEM (prewarmed to 37°) containing 5% FBS or CS and fluorescent substrate with or without reversing agent for 40 min at 37° in 6 mL polystyrene tubes with caps. The following concentration of fluorescent compounds can be used: rhodamine-123, 0.5 μg/mL; bodipy-verapamil, 0.5 μM (Molecular Probes, Eugene, OR); calcein AM, 0.5 μM (Molecular Probes); daunomycin, 4 μM (Calbiochem). Measurements of calcein AM accumulation are taken after a 10-min incubation at 37°.
2. Centrifuge cells at 200*g* for 5 min and remove medium.
3. Resuspend the cells in 300 μl ice-cold PBS and analyze by FACS.
4. For rhodamine-123 and daunomycin measurements, resuspend cells in 4.5 mL substrate-free medium with or without reversing agent.
5. Incubate an additional 40 min at 37°.
6. Pellet the cells by centrifugation at 200*g* for 5 min and remove the medium.
7. Resuspend the cells in 450 μl ice-cold PBS and analyze by FACS.

Drug Accumulation Assays Using Radiolabeled Compounds in Intact Cells

Drug accumulation assays in infected–transfected cells can be performed essentially as described by Stein *et al.*[20] However, for experimental

[20] W. D. Stein, C. O. Cardarelli, I. Pastan, and M. M. Gottesman, *Mol. Pharmacol.* **45,** 763 (1994).

ease and reproducibility of results, the modified protocol described in detail next has been developed allowing for the assay to be performed with cells in suspension.

Materials

Iscove's modified Dulbecco's medium (IMEM) (Life Technologies)
Trypsin-EDTA: 0.25% w/v trypsin, 1 m*M* EDTA
[^{3}H]Colchicine (74.0 Ci/mmol) (DuPont-NEN)
[^{3}H]Vinblastine (13.5 Ci/mmol) (Amersham Life Science)
[^{3}H]Daunomycin (4.4 Ci/mmol) (DuPont-NEN)
Formula-989 (NEF-989; Packard, Downers Grove, IL) liquid scintillation cocktail
Phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS)
6-mL 12 × 75-mm polystyrene round-bottom tubes with caps (Falcon)

Method

1. Infect–transfect 70–80% confluent HOS or HeLa cells in 75-cm^2 flasks.
2. Remove cells from flasks 16–24 hr after infection–transfection by trypsinization and wash once in IMEM containing 5% FBS or CS.
3. To initiate the drug uptake, incubate 500,000 cells in 4 ml of IMEM containing 55 mM glucose and radioactive substrate for 50 min at 37° in 6-mL polystyrene tubes with caps. The following concentrations of radioactive substrates can be used: [^{3}H]colchicine (100 n*M*, 0.5 μCi/ml), [^{3}H]vinblastine (13.5 n*M*, 0.25 μCi/ml), or [^{3}H]daunomycin (113.5 n*M*, 0.5 μCi/ml). Other radioactively labeled compounds can also be used. To determine specificity or efficacy, assays should also be performed in the presence of a reversing agent or inhibitors. Assays should all be performed in duplicate or triplicate.
4. Gently resuspend cells in 500 μL ice-cold serum-free and glucose-free IMEM and add an additional 4 mL of the same medium to wash the cells.
5. Centrifuge cells at 200*g* for 5 min and remove medium.
6. For determining the nonspecific binding of drug to cells, add ice-cold IMEM containing the labeled drug and centrifuge immediately at 200*g* for 5 min. Remove the medium and wash the cells as described earlier keeping the cells on ice at all times.
7. Transfer cells with 400 μL of cold PBS to scintillation vials containing 15 ml of a high-flash point liquid scintillation cocktail such as Formula-989. Rinse the tube with an additional 400 μL PBS and count total radioactivity using a liquid scintillation counter.
8. Express the intracellular accumulation as picomoles of labeled drug per million cells.

Conclusions

The vaccinia-T7 expression system has proven to be ideal for functional studies of Pgp, the transporter associated with multidrug resistance (MDR). Previously, other heterologous expression systems have been explored but none have been completely satisfying.[1,21] Of those examined the baculovirus/insect cell system appears to be most promising for the large-scale synthesis of protein for biochemical and structural analysis. However, whole-cell functional assays are not feasible because the infection process appears to deplete the intracellular ATP levels and make the cell membrane leaky to drugs.[21] Expression of human Pgp in the yeast *Saccharomyces cerevisiae* and *E. coli* have met with limited success owing to low expression levels, toxicity, or intrinsically high ATPase levels, but neither has proven as versatile as the vaccinia-T7 system.[1,10,21,22] Higher level expression in the yeast *Pichia pastoris* appears to be feasible.[23] Historically, in order to express large amounts of protein in mammalian cells, it has been necessary to establish stably transfected cell lines selected stepwise in MDR drugs. Because this process can take weeks to months, these selection schemes are a constant source of debate because of the unknown pleiotropic effects of drug selection on cellular functions. The use of this vaccinia-T7 transient system that does not involve drug selection for Pgp expression eliminates the need to consider these possible complications in the interpretation of the observed phenotypes.

The major drawback of the vaccinia-T7 system is that the infected–transfected cells cannot be used to measure relative resistance to MDR drugs in cell proliferation assays since the infected cells are committed to virus-induced lysis. In the future, it may be possible to make use of a non- or reduced-cytopathic vaccinia virus (MVA-Pol)[24] that will allow for use in these types of assays. However, currently, changes in substrate specificity resulting from mutations can be examined with transport assays using fluorescent or radiolabeled compounds with vTF7-3 infected–transfected cells. Another system that appears to have some promise utilizes the canarypox virus, which has the advantage of not inhibiting host-cell replication[25,26] suggesting that these infected cells would remain targets for cytotoxic drugs.

[21] G. L. Evans, B. Ni, C. A. Hrycyna, D. Chen, S. V. Ambudkar, I. Pastan, U. A. Germann, and M. M. Gottesman, *J. Bioenerg. Biomembr.* **27,** 43 (1995).

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[25] J. Taylor, R. Weinberg, J. Tartaglia, C. Richardson, G. Alkhatib, D. Briedis, M. Appel, E. Norton, and E. Paoletti, *Virology* **187,** 321 (1992).

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