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Cancer Cell Signaling

Methods and Protocols

Edited by

David M. Terrian

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Cancer Cell Signaling

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Preface

Cells respond to environmental cues through a complex and dynamic network of signaling pathways that normally maintain a critical balance between cellular proliferation, differentiation, senescence, and death. One current research challenge is to identify those aberrations in signal transduction that directly contribute to a loss of this division-limited equilibrium and the progression to malignant transformation. The study of cell-signaling molecules in this context is a central component of cancer research. From the knowledge of such targets, investigators have been able to productively advance many insightful hypotheses about how a particular cancer cell may misinterpret, or respond inappropriately to, growth regulatory cues in their environment. Despite these key insights, the rapidly evolving nature of cell signaling research in cancer has necessitated a continuous revision of these theoretical constructs and the updating of methods used in their study. One contemporary example of the evolution of this field is provided by an analysis of the Human Genome Project data, which reveal a previously unsuspected diversity in the multigene families encoding for most signaling pathway intermediates. In assessing the usefulness of a particular methodological approach, therefore, we will need to keep in mind that there is a premium on those protocols that can be easily adapted for the analysis of multiple members within a gene family. *Cancer Cell Signaling: Methods and Protocols* brings together several such methods in cell signaling research that are scientifically grounded within the cancer biology field. The first part of this volume is generally concerned with methods and techniques for the investigation of apoptosis and cell death. The second part contains a complementary set of protocols for manipulating and/or monitoring oncogenic signals in cancer cells. In the third, methods for studying protein–protein interactions are covered. Finally, in part four, there is a detailed protocol for capturing pure samples of malignant cells from frozen tissue specimens and two alternative techniques for analyzing their genomic DNA.

I thank the authors for providing such clear and detailed accounts of their experimental protocols and for the many useful hints they have generously included in the notes to each chapter.

David M. Terrian

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I

Manipulation and Detection of Survival Signals

1

Functional Analysis of the Antimitogenic Activity of Tumor Suppressors

Erik S. Knudsen and Steven P. Angus

Abstract

Loss of tumor suppressors contributes to numerous cancer types. Many, but not all, proteins encoded by tumor suppressor genes have antiproliferative activity and halt cell-cycle progression. In this chapter, we present three methods that have been utilized to monitor the antimitogenic action exerted by tumor suppressors. Tumor suppressor function can be demonstrated by colony formation assays and acquisition of the flat-cell phenotype. Because of the antiproliferative action of these agents, we also present two transient assays that monitor the effect of tumor suppressors on cell-cycle progression. One is based on BrdU incorporation (i.e., DNA replication) and the other on flow cytometry. Together, this triad of techniques is sufficient to determine the action of tumor suppressors and other antiproliferative agents.

Key Words: Tumor suppressor; green fluorescent protein; bromo-deoxyuridine; retinoblastoma; cell cycle; cyclin; flow cytometry; mitogen; fluorescence microscopy.

1. Introduction

The discovery of tumor suppressor genes, whose loss predisposes to tumor development, has revolutionized the molecular analysis of cancer ***(1–3)***. By definition, tumor suppressor genes are genetically linked to a cancer. For example, the retinoblastoma (RB) tumor suppressor was first identified as a gene that was specifically lost in familial RB ***(4–6)***. The majority of tumor suppressors

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has been identified based on linkage analysis and subsequent epidemiological studies, however, initial understanding of their mode of action was relatively limited. As the number of tumor suppressors has increased, understanding the mechanism through which tumor suppressors function has become an important aspect of cancer biology.

In general, tumors exhibit uncontrolled proliferation. This phenotype can arise from loss of tumor suppressors that regulate progression through the cell cycle (e.g., RB or p16ink4a) or upstream mitogenic signaling (e.g., NF1 or PTEN) cascades ***(1,3,7–9)***. Thus, specific tumor suppressors can function to suppress proliferation. However, not all tumor suppressors act in this manner. For example, mismatch repair factors (e.g., MSH2 or MLH-1) lost in hereditary nonpolyposis colorectal cancer (HNPCC) function not to inhibit proliferation, but to prevent further mutations ***(10–12)***. Additionally, other tumor suppressors have multiple functions, for example, *p53* can function to either induce cell death or halt cell-cycle progression ***(9,13)***.

Functional analysis of tumor suppressors relies on a host of methods to determine how or if they inhibit proliferation. Later, we will focus on methods that have been used to assess the antimitogenic potential of the RB-pathway ***(2,3,7, 14)***. However, these same approaches are amenable to any tumor suppressor or antimitogenic molecule.

Assays used to evaluate antimitogenic activity are based either on the halt of proliferation or cell-cycle progression. Cell proliferation assays, as described later, have been extensively utilized to demonstrate the antiproliferative effect of tumor suppressors ***(15–20)***. However, these assays do not illuminate whether the observed effects are attributable to cell-cycle arrest or apoptosis. Additionally, because of the antiproliferative action of many tumor suppressors, it is difficult to obtain sufficient populations of cells for analysis. This obstacle can be surmounted through the use of transient assays to monitor cell-cycle effects ***(16,19,21–25)***. Two different transient approaches to analyze tumor suppressor action on the cell cycle are also described.

2. Materials

2.1. Cell Culture and Transfection of Antimitogen/Tumor Suppressor

1. SAOS-2 human osteosarcoma cell line (ATCC #HTB-85).
2. Dulbecco's modification of Eagle's medium (DMEM, Cellgro, cat #10-017-CV) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Atlanta Biologicals, cat #S12450), 100 U/mL penicillin-streptomycin and 2 m*M* L-glutamine (Gibco-BRL).

3. Dulbecco's phosphate-buffered saline (PBS), tissue culture grade, without calcium and magnesium (Cellgro, cat #21-031-CV).
4. 1X Trypsin-EDTA solution (Cellgro, cat #25-052-CI).
5. 60-mm tissue-culture dishes.
6. Six-well tissue-culture dishes.
7. 12-mm circular glass cover slips (Fisher), sterilized.
8. Mammalian expression system (e.g., pcDNA3.1, Invitrogen).
9. Relevant cDNAs: RB, Histone 2B (H2B)-GFP [from G. Wahl, The Salk Institute, La Jolla, CA ***(26)***], pBABE-puro [puromycin resistance plasmid, ***(27)***].
10. 0.25M $CaCl_2$: dissolve in ddH_2O; filter (0.2 μm) sterilize and store in aliquots at –20ºC.
11. 2X BES-buffered solution (2X BBS): 50 m*M* N,N-*bis* (2-hydroxyethyl)-2-amino-ethanesulfonic acid, 280 m*M* NaCl, 1.5 m*M* Na_2HPO_4, adjust pH to 6.95 in ddH_2O, filter (0.2 μm) sterilize and store in aliquots at –20ºC.
12. Inverted fluorescence microscope (Zeiss).

2.2. Inhibition of BrdU Incorporation in Transiently-Transfected Cells

1. Transfected SAOS-2 cells.
2. Cell proliferation-labeling reagent, BrdU/FdU (Amersham Pharmacia, cat# RPN201).
3. PBS: 136 m*M* NaCl, 2.6 m*M* KCl, 10m*M* Na_2HPO_4, 2.7 m*M* KH_2PO_4 in ddH_2O; pH to 7.4 with HCl; sterilize in autoclave.
4. 3.7% (v/v) formaldehyde in PBS: dilute fresh from 37% w/w stock solution (Fisher).
5. 0.3% (v/v) Triton X-100 (Fisher) in PBS.
6. Immunofluorescence (IF) buffer: 0.5% v/v Nonidet P-40 (Fisher) and 5 mg/mL (w/v) bovine serum albumin (Sigma) in PBS; store at 4ºC.
7. 1*M* $MgCl_2$.
8. DNase I, RNase-free (10 U/μL) (Roche, cat# 776 785).
9. Monoclonal rat anti-BrdU antibody (Accurate Scientific, cat #YSRTOBT-0030).
10. Donkey anti-rat IgG, Red X-conjugated (Jackson Immunoresearch, cat #712-295-153).
11. 1 mg/mL (w/v) Hoechst 33258 (Sigma, cat #B2883).
12. Microscope slides.
13. Gel/Mount (Biomeda Corp., cat #MØ1)
14. Inverted fluorescence microscope (Zeiss).

2.3. Cell-Cycle Analysis of Transiently-Transfected Cells

1. Transfected SAOS-2 cells.
2. PBS.
3. 1X Trypsin-ethylene diamine tetraacetic acid (EDTA) solution (Cellgro, cat #25-052-CI).
4. Clinical centrifuge.

5. 100% ethanol stored at –20ºC.
6. 40 mg/mL (w/v) RNase A (Sigma, cat #R-4875): Dissolve in sterile double-distilled (dd)H_2O at 100ºC, 15 min; aliquot and store at –20ºC.
7. 100X propidium iodide (PI) solution: 20 mg/mL (w/v) propidium iodide (Sigma, cat #P-4170) in PBS; cover with foil to protect from light and store at 4ºC.
8. 5-mL polystyrene round-bottom tubes (Becton Dickinson, cat #35-2058).
9. Coulter Epics XL flow cytometer.
10. FlowJo data analysis software (Treestar).
11. ModFit cell-cycle analysis software (Verity).

2.4. Flat-Cell Assay and Colony Inhibition in Stably-Transfected Cells

1. Transfected SAOS-2 cells.
2. 2.5 mg/mL puromycin (w/v) (Sigma, cat #P-7255).
3. 1% crystal violet (w/v) (Fisher, cat #C581-25)/20% ethanol solution.
4. Inverted microscope with camera.

3. Methods

3.1. Cell Culture and Transfection of Antimitogen/Tumor Suppressor

3.1.1. Cell Culture

1. Seed approx 1×10^5 cells per well of a six-well plate or 3×10^5 cells per 60-mm dish in DMEM supplemented with 10% FBS and penicillin-streptomycin.
2. SAOS-2 cells should attach to the tissue culture dish within 4–6 h.

3.1.2. Cell Transfection

1. Prepare purified plasmid DNA stocks at 1 mg/mL concentration in TE buffer.
2. Add DNA to 1.5-mL Eppendorf tube (4.25 µg per well of a six-well plate, 8.5 µg total per 60-mm dish).
3. Add 0.25*M* $CaCl_2$ to DNA and mix by pipeting.
4. Add 2X BBS solution and mix by inverting.
5. Incubate tubes at room temperature for 20 min.
6. Add DNA/$CaCl_2$/BBS solution to cells dropwise.
7. Inspect the cells for the presence of precipitate using an inverted microscope (20× power is sufficient) (*see* **Note 1**).
8. Return cells to tissue culture incubator (37ºC, 5% CO_2).
9. 16 h postaddition of precipitate, wash cells three times briefly with PBS.
10. Inspect dishes to ensure removal of precipitate.
11. Add fresh media to cells.

3.1.3. Confirmation of Transfection/ Determining Transfection Efficiency

1. Take live plates of cells transfected 16 h prior with H2B-GFP and either vector or antimitogen/tumor suppressor out of the incubator.
2. Aspirate media.
3. Replace with PBS.
4. Visualize transfected cells by GFP fluorescence using an inverted fluorescent microscope (20X power is sufficient).
5. Using the GFP fluorescence and phase contrast, determine the percentage of GFP-positive cells by counting random fields of cells.
6. Compare the relative transfection efficiencies between vector control and antimitogen/tumor suppressor.

3.2. Inhibition of BrdU Incorporation in Transiently Transfected Cells

3.2.1. Cell Culture

1. Culture cells at 60% confluence (approx 1×10^5 cells/well) on coverslips in a six-well plate (four cover slips per well).

3.2.2. Cell Transfection

1. Use 4 µg of CMV-vector or CMV-RB and 0.25 µg of CMV-H2B-GFP.
2. Use 0.125 mL $CaCl_2$ and 0.125 mL 2X BBS.

3.2.3. BrdU Labeling

1. 36–48 h after adding fresh media to transfected cells, add cell proliferation-labeling reagent directly to media in wells (1:1000 dilution) (*see* **Note 2**).
2. Return six-well dish to tissue-culture incubator for 16 h.

3.2.4. Fixation

1. Aspirate media from wells.
2. Wash cells gently with PBS.
3. Fix cells at room temperature with 3.7% formaldehyde in PBS for 15 min.
4. Aspirate formaldehyde.
5. Add PBS to wells.
6. Cover slips in PBS may be stored in dark at 4ºC.

3.2.5. BrdU Staining

1. Aspirate PBS.
2. Add 0.3% Triton X-100 in PBS to wells to permeabilize the cells (*see* **Note 3**).
3. Incubate dish at room temperature for 15 min.
4. Aspirate 0.3% Triton X-100 and replace with PBS.

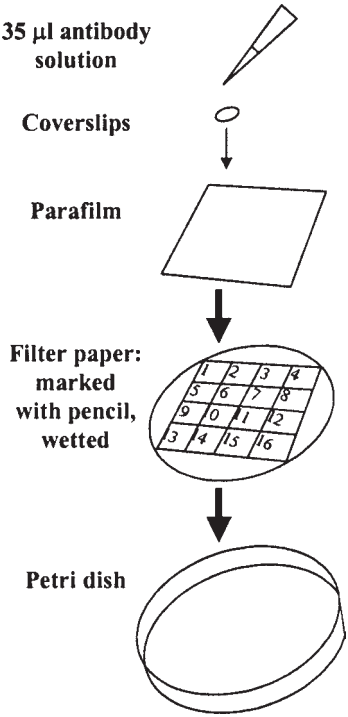

Fig. 1. Diagram of BrdU staining in a humidified chamber of fixed and permeabilized cells grown on glass cover slips.

5. Prepare primary antibody solution by diluting the following in IF buffer:
   a. 1:50 1M $MgCl_2$.
   b. 1:500 Rat anti-BrdU.
   c. 1:500 DNase I (*see* **Note 4**).
6. Pipet 35 µL primary antibody solution onto each cover slip.
7. Incubate cover slips in a humidified chamber at 37ºC for 45 min (*see* **Fig. 1**).
8. Wash cover slips in PBS in six-well dish for 5 min with 2–3 changes.
9. Prepare secondary antibody solution by diluting the following in IF buffer:
   a. 1:100 Donkey anti-rat Red-X.
   b. 1:100 Hoechst (10 µg/mL final conc.).
10. Pipet 35 µL secondary antibody solution onto each cover slip.
11. Incubate cover slips in humidified chamber at 37ºC for 45 min.
12. Wash cover slips in PBS in six-well dish for 5 min with 2–3 changes.
13. Mount cover slips on slides using Gel/Mount.
14. Examine cover slips using an inverted fluorescence microscope.
15. Inhibition determined by counting.

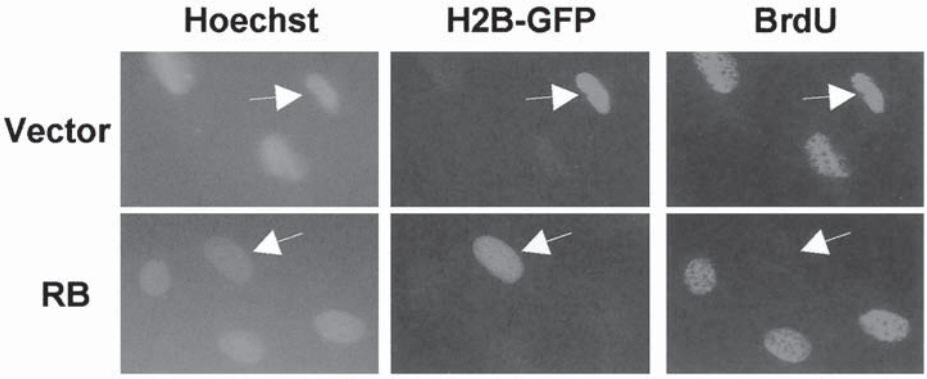

Fig. 2. SAOS-2 cells were cotransfected with H2B-GFP and either CMV-vector or CMV-RB. Cells were pulse-labeled with BrdU for 16 h. Fixation, permeabilization, and immunostaining were performed as described. Photomicrographs of immunofluorescent cells were taken at equal magnification. Arrows indicate transfected cells. Quantification of this approach is presented in **refs.** ***(19,21–23)***.

3.2.6. Quantitation and Documentation

1. Quantitation of BrdU inhibition.
   a. Count the number of transfected (i.e., GFP-positive) cells in a random field).
   b. Without changing fields, count the number of GFP-positive cells that are also BrdU-positive (i.e., Red-X-positive).
   c. Repeat **steps a** and **b** until 150–200 GFP-positive cells have been counted.
   d. Calculate the percent BrdU-positive (BrdU-positive/GFP-positive).
   e. As a control, determine the percentage of BrdU-positive cells from untransfected (GFP-negative) cells on the same cover slips.
   f. Compare the effect of antimitogen expression vs vector expression on BrdU incorporation (*see* **Fig. 2**).
2. Documentation
   a. Take representative photomicrographs of selected fields.
   b. Use blue (Hoechst), green (H2B-GFP), and red (Red-X) channels to obtain photomicrographs of the same field.

3.3. Cell-Cycle Arrest in Transiently-Transfected Cells

3.3.1. Cell Culture

1. Culture cells in 60-mm dishes at 60% confluence.
2. Include a dish that will not be transfected.

3.3.2. Cell Transfection

1. Use 8 µg of CMV-vector or CMV-RB and 0.5 µg of CMV-H2B-GFP (*see* **Note 5**).
2. Use 0.25 mL $CaCl_2$ and 0.25 mL 2X BBS.

3.3.3. Cell Harvesting and Fixation

1. 36–48 h after adding fresh media to transfected cells, add trysin (approx 0.75 mL) to dishes.
2. Confirm that cells have detached after 1–2 min using inverted microscope.
3. Inactivate trypsin by adding an equal volume of media.
4. Transfer suspended cells to 15-mL conical tubes.
5. Pellet cells in a clinical centrifuge at 1000 rpm, 2–3 min.
6. Aspirate media.
7. Add 2–3 mL PBS to wash cell pellet.
8. Repeat centrifugation.
9. Aspirate PBS.
10. Resuspend cell pellet in 200 µL PBS.
11. Slowly add 1 mL ice-cold 100% ethanol while vortexing gently.
12. Tubes may be stored in the dark at 4ºC for 1–2 wk.

3.3.4. Propidium Iodide Staining

1. Prepare 1X PI by diluting 100X PI stock solution in PBS (*see* **Note 6**).
2. Add RNase A to 1X PI at a 1:1000 dilution (final concentration = 40 µg/mL).
3. Pellet fixed cells at 200*g*, 2–3 min.
4. Aspirate ethanol.
5. Resuspend cell pellet in approx 1 mL 1X PI containing RNase A.
6. Transfer resuspended cells to 5-mL polystyrene round-bottom tubes.
7. Incubate tubes in the dark at room temperature for at least 15 min prior to analysis (*see* **Note 7**).

3.3.5. FACS

1. Run untransfected control to set background levels of GFP signal and to establish PI parameters.
2. Gate H2B-GFP-positive cells (either positive or negative) (*see* **Fig. 3** and **Note 8**).
3. Analyze PI staining in GFP-positive cells.
4. Perform ModFit analysis on PI histograms (*see* **Fig. 3**).

3.4. Flat-Cell Assay/Colony Inhibition in Stably Transfected Cells

3.4.1. Cell Culture

1. Culture 1×10^5 cells in 60-mm dishes.
2. Include a control plate that will not be transfected.

3.4.2. Cell Transfection

1. Use 8 µg of CMV-vector or CMV-RB and 0.5 µg of pBABE-puro.
2. Use 0.25 mL $CaCl_2$ and 0.25 mL 2X BBS.

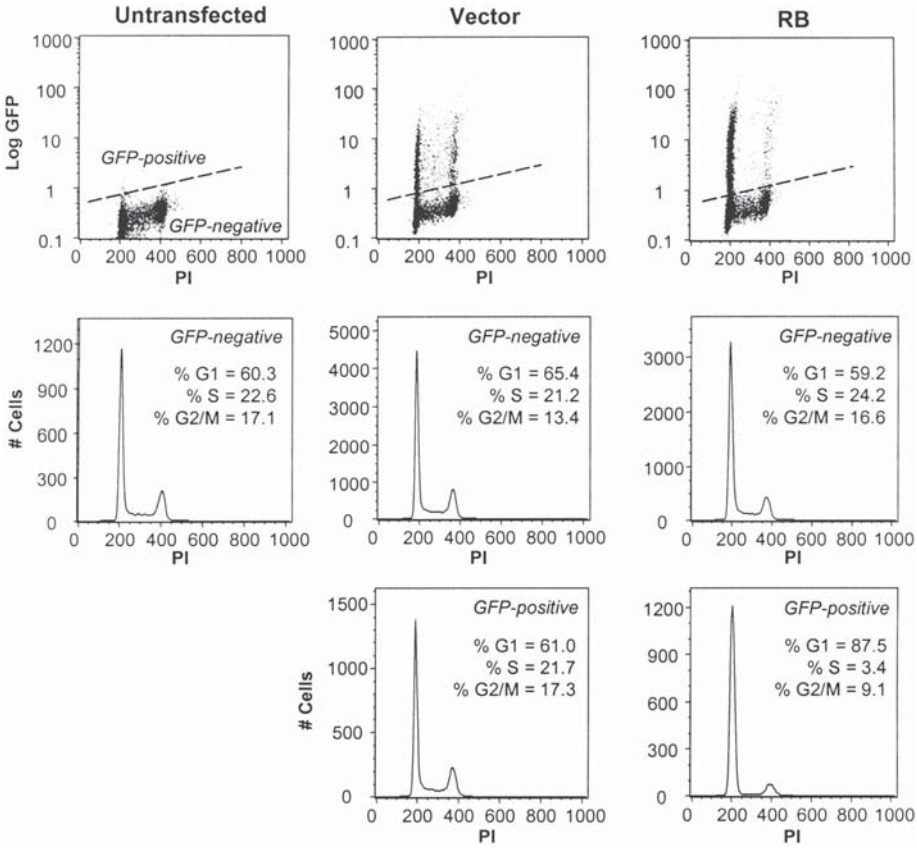

Fig. 3. SAOS-2 cells either untransfected (*left column*) or transfected with H2B-GFP and either CMV-vector (*middle column*), or RB (*right column*) were fixed in ethanol and stained with propidium iodide (PI). Cells were subsequently analyzed by FACS. *Top row*, Cells were gated to distinguish the GFP-negative population from the GFP-positive population. Hatched line indicates gate position (GFP-positive cells above line, GFP-negative cells below). *Middle row*, GFP-negative cells were analyzed for DNA content (PI) and ModFit analysis was performed to quantitate cell cycle distribution (% phase) as indicated. *Bottom row*, GFP-positive cells were analyzed for DNA content (PI) and ModFit analysis was performed to quantitate cell cycle distribution (% phase) as indicated.

3.4.3. Puromycin Selection and Staining

1. 24 h after adding fresh media to transfected cells, add puromycin to media at a 1:1000 dilution (final concentration = 2.5 µg/mL puromycin).
2. Confirm puromycin selection by visual analysis of untransfected cells.

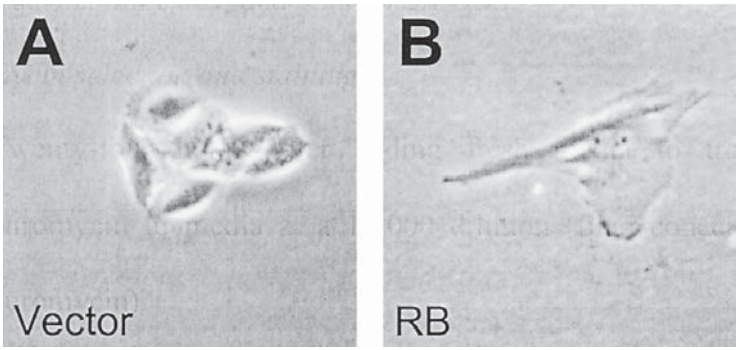

Fig. 4. SAOS-2 cells transfected as described with pBABE-puro and either (**A**) CMV-vector or (**B**) RB were selected with 2.5 µg/mL puromycin for 4 d. Note the flat-cell phenotype exhibited by the RB-transfected cell. Phase-contrast photomicrographs are of equal magnification. Quantitation of this approach and colony outgrowth is published in **refs.** ***(15–17,19,20,25)***.

3. Monitor selection/cell death daily by visual analysis using an inverted microscope (*see* **Fig. 4**).
4. Plates for flat-cell analysis should be stained 5–8 d postselection.
5. Plates for colony inhibition should be analyzed 8–14 d postselection.

3.4.4. Crystal Violet Staining

1. Aspirate media.
2. Wash plates twice with PBS.
3. Add 5 mL 1% crystal violet/20% ethanol solution to cell plates.
4. Incubate plates at room temperature 5 min.
5. Immerse plates in ice-cold water bath.
6. Rinse until no more crystal violet is washing into the water.
7. Invert plates on paper towels and dry at room temperature.
8. Dried plates will store for greater than 1 yr kept in the dark.

3.4.5. Quantitation and Documentation

1. Flat-cell phenotype
   a. Using a microscope with a grid of known unit area, count flat cells present in multiple random fields.
   b. To document results, take low-magnification (×10 or ×20) pictures of the flat cells (*see* **Fig. 4**).
2. Colony inhibition
   a. Count all visible colonies on plate or in a specific unit area of the plate.
   b. To document results, take a picture of the entire plate (no magnification required).

4. Notes

1. The formation/presence of black, granular precipitate ensures the quality of the transfection reagents (i.e., 0.25*M* $CaCl_2$ and 2X BBS). Poor precipitate formation is often due to incorrect pH of the 2X BBS solution.
2. BrdU is light sensitive. Add to tissue-culture dishes in the dark, and limit light exposure (as with fluorophores) during staining.
3. We typically permeabilize and stain only one or two of the fixed coverslips from each well, in case of errors during staining.
4. We recommend using DNase I only from Roche. DNase I purchased from other companies has produced poor results, likely because of excess enzyme activity.
5. Cotransfection with H2B-GFP fusion protein (as opposed to GFP alone) to distinguish transfected cells is essential particularly for FACS anaylsis. The use of ethanol to fix cells for propidium iodide staining results in the loss of soluble protein. However, other markers (e.g., CD20; *see* **ref.** ***(25)***) that provide green fluorescent signal for sorting may be used.
6. Propidium iodide is light sensitive. Stock solution and resuspended cells in 1X PI should be protected from light with foil.
7. Adequate incubation time to allow complete RNase digestion is critical for interpretable results.
8. The percentage of GFP-positive cells determined by FACS analysis should be approximately equal to the percentage determined by visual inspection prior to harvesting.

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References

1. Knudson, A. G. (1993) Antioncogenes and human cancer. *Proc. Natl. Acad. Sci. USA* **90,** 10,914–10,921.
2. Weinberg, R. A. (1995) The retinoblastoma protein and cell cycle control. *Cell* **81,** 323–30.
3. Macleod, K. (2000) Tumor suppressor genes. *Curr. Opin. Genet. Dev.* **10,** 81–93.
4. Cavenee, W. K., Dryja, T. P., Phillips, R. A., Benedict, W. F., Godbout, R., Gallie, B. L., et al. (1983) Expression of recessive alleles by chromosomal mechanisms in retinoblastoma. *Nature* **305,** 779–784.
5. Friend, S. H., Bernards, R., Rogelj, S., Weinberg, R. A., Rapaport, J. M., Albert, D. M., and Dryja, T. P. (1986) A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. *Nature* **323,** 643–646.

6. Lee, W. H., Bookstein, R., Hong, F., Young, L. J., Shew, J. Y., and Lee, E. Y. (1987) Human retinoblastoma susceptibility gene: cloning, identification, and sequence. *Science* **235,** 1394–1329.
7. Sherr, C. J. (1996) Cancer cell cycles. *Science* **274,** 1672–1677.
8. Hanahan, D. and Weinberg, R. A. (2000) The hallmarks of cancer. *Cell* **100,** 57–70.
9. Evan, G. I. and Vousden, K. H. (2001) Proliferation, cell cycle and apoptosis in cancer. *Nature* **411,** 342–348.
10. Peltomaki, P. (2001) Deficient DNA mismatch repair: a common etiologic factor for colon cancer. *Hum. Mol. Genet.* **10,** 735–740.
11. Kolodner, R. D. (1995) Mismatch repair: mechanisms and relationship to cancer susceptibility. *Trends Biochem. Sci.* **20,** 397–401.
12. Kinzler, K. W. and Vogelstein, B. (1996) Lessons from hereditary colorectal cancer. *Cell* **87,** 159–170.
13. Levine, A. J. (1997) p53, the cellular gatekeeper for growth and division. *Cell* **88,** 323–331.
14. Wang, J. Y., Knudsen, E. S., and Welch, P. J. (1994) The retinoblastoma tumor suppressor protein. *Adv. Cancer Res.* **64,** 25–85.
15. Arap, W., Knudsen, E., Sewell, D. A., Sidransky, D., Wang, J. Y., Huang, H. J., and Cavenee, W. K. (1997) Functional analysis of wild-type and malignant glioma derived CDKN2Abeta alleles: evidence for an RB-independent growth suppressive pathway. *Oncogene* **15,** 2013–2020.
16. Arap, W., Knudsen, E. S., Wang, J. Y., Cavenee, W. K., and Huang, H. J. (1997) Point mutations can inactivate in vitro and in vivo activities of p16(INK4a)/CDKN2A in human glioma. *Oncogene* **14,** 603–609.
17. Hinds, P. W., Mittnacht, S., Dulic, V., Arnold, A., Reed, S. I., and Weinberg, R. A. (1992) Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. *Cell* **70,** 993–1006.
18. Templeton, D. J., Park, S. H., Lanier, L., and Weinberg, R. A. (1991) Nonfunctional mutants of the retinoblastoma protein are characterized by defects in phosphorylation, viral oncoprotein association, and nuclear tethering. *Proc. Natl. Acad. Sci. USA* **88,** 3033–3037.
19. Knudsen, K. E., Weber, E., Arden, K. C., Cavenee, W. K., Feramisco, J. R., and Knudsen, E. S. (1999) The retinoblastoma tumor suppressor inhibits cellular proliferation through two distinct mechanisms: inhibition of cell cycle progression and induction of cell death. *Oncogene* **18,** 5239–45.
20. Qin, X. Q., Chittenden, T., Livingston, D. M., and Kaelin, W. G. Jr. (1992) Identification of a growth suppression domain within the retinoblastoma gene product. *Genes Dev.* **6,** 953–964.
21. Knudsen, E. S., Pazzagli, C., Born, T. L., Bertolaet, B. L., Knudsen, K. E., Arden, K. C., et al. (1998) Elevated cyclins and cyclin-dependent kinase activity in the rhabdomyosarcoma cell line RD. *Cancer Res.* **58,** 2042–2049.
22. Knudsen, E. S., Buckmaster, C., Chen, T. T., Feramisco, J. R., and Wang, J. Y. (1998) Inhibition of DNA synthesis by RB: effects on G1/S transition and S-phase progression. *Genes Dev.* **12,** 2278–2292.

23. Knudsen, K. E., Fribourg, A. F., Strobeck, M. W., Blanchard, J. M., and Knudsen, E. S. (1999) Cyclin A is a functional target of retinoblastoma tumor suppressor protein-mediated cell cycle arrest. *J. Biol. Chem.* **274,** 27,632–27,641.
24. Agami, R. and Bernards, R. (2000) Distinct initiation and maintenance mechanisms cooperate to induce G1 cell cycle arrest in response to DNA damage. *Cell* **102,** 55–66.
25. Zhu, L., van den Heuvel, S., Helin, K., Fattaey, A., Ewen, M., Livingston, D., et al. (1993) Inhibition of cell proliferation by p107, a relative of the retinoblastoma protein. *Genes Dev.* **7,** 1111–1125.
26. Kanda, T., Sullivan, K. F., and Wahl, G. M. (1998) Histone-GFP fusion protein enables sensitive analysis of chromosome dynamics in living mammalian cells. *Curr. Biol.* **8,** 377–385.
27. Morgenstern, J. P. and Land, H. (1990) Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Res.* **18,** 3587-3596.

2

Rescue and Isolation of *Rb*-deficient Prostate Epithelium by Tissue Recombination

Simon W. Hayward, Yuzhuo Wang, and Mark L. Day

Abstract

The ability to rescue viable prostate precursor tissue from *Rb–/–* fetal mice has allowed for the generation of *Rb–/–* prostate tissue and *Rb–/–* prostate epithelial cell lines. Herein, we provide a protocol for the rescue of urogenital precursor tissue from mouse embryos harboring the lethal *Rb–/–* mutation. The rescued precursors can matured as subrenal capsule grafts in athymic mice. Subsequently prostatic tissue can be used as a source for *Rb–/–* epithelium in a tissue recombination protocol for the generation of chimeric prostate grafts in athymic male mouse hosts. We have also provided a detailed description for isolating and propagating the *Rb–/–* epithelium from such tissue recombinants as established cell lines. Methods for characterizing the grafts and cell lines by determining the retention of prostate-specific epithelial expression markers, including cytokeratins, the androgen receptor, estrogen receptor β and the dorsolateral prostatic secretory protein (mDLP) are given.

Key Words: Retinoblastoma (*Rb*); primary culture; development; genotyping; tissue recombination (TR); prostate; epithelium; differentiation; immortalization.

1. Introduction

Prostate carcinogenesis is a multistep process involving the perturbation of normal stromal-epithelial interactions ***(1–3)*** and genetic alterations of the epithelium resulting in activation of oncogenes ***(4–7)*** and inactivation of tumor-suppressor

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genes ***(8,9)***. The involvement of multiple oncogenes and tumor-suppressor genes in carcinogenesis has been demonstrated in many types of human carcinomas ***(10,11)***. Alterations in tumor-suppressor genes such as the retinoblastoma (*Rb*) gene have been suggested to play a role in the development of human prostate cancer ***(8,12–14)***. The *Rb* gene encodes a 110 kDa phosphoprotein (pRb) that regulates the transition between G1 and S phases in the cell cycle by transducing growth-inhibitory signals that arrest cells in G1 ***(15)***. Functional regulation of pRb is cell-cycle dependent, being strictly controlled by the activity of cyclin-dependent kinases that regulate the state of pRb phosphorylation. The growth inhibitory function of pRb is attained through signals exerted at the level of gene transcription in association with the E2F family of transcription factors. As the cell approaches the G1-S border, pRb can be sequentially phosphorylated (inactivated) by cyclin D/cdk4/6 and cyclin E/cdk2 complexes, leading to the release of E2F and subsequent activation of E2F-regulated genes that are required for S-phase entry ***(16)***. The importance of the *Rb* gene in tumorigenesis was originally recognized in familial retinoblastoma and subsequently the involvement of *Rb* has been described in many human cancers including bladder ***(17)***, breast ***(18–20)***, and lung cancer ***(21–23)***. In human prostate cancer, estimates of the frequency of *Rb* gene mutations and deletions vary widely covering a range from 1–50% of cancer cases ***(24–32)***. To some extent this disparity may be a result of *Rb* alterations being infrequent in early human prostate cancer and becoming more common as the disease progresses. However, a review of the literature still shows disparities between estimates at apparently matched disease stages. It is clear though that a subset of human prostate cancer does contain changes at the *Rb* gene and protein levels. The function of *Rb* and its role in human carcinogenesis has been the subject of vigorous investigation for a number of years, however, the specific role *Rb* plays in the etiology of prostate cancer has yet to be determined.

A major obstacle to the investigation of *Rb* in carcinogenesis has been the lethality of the homozygous *Rb* knockout in mice. Mice homozygous for *Rb* disruption (*Rb*–/–) die at 13 d of gestation, several days before the prostate forms. The cause of death, disruption of erythropoiesis and neurogenesis, is unrelated to many of the tumors that could usefully be studied using these animals. At first sight, it would appear problematic to study prostatic carcinogenesis in mice that die before prostatic tissue forms. We have recently overcome this obstacle and have been able to circumvent the lethal phenotype through the employment of tissue rescue and recombination technology ***(33)***. Tissue rescue involves grafting organs, or organ precursors, beneath the renal capsule of athymic rodent hosts where they can undergo development to form the tissues of interest. Tissue recombination allows amplification of specific epithelial cell populations from the rescued tissues. This procedure has enabled the isolation of *Rb*–/– prostate

tissues and cells. In this model, pelvic visceral rudiments of E12 *Rb–/–* embryos were grown as subrenal capsule grafts in adult male nude mouse hosts. Following a month of development, prostatic tissue was microdissected and characterized. *Rb–/–* prostatic epithelial cells were then expanded by recombining prostatic ductal tips from the microdissected tissue with rat urogenital sinus mesenchyme and regrafting the resultant recombinants to new male athymic mouse hosts ***(33)***. These grafts have been shown to retain multiple molecular markers of prostate epithelium as well as sensitivity to hormones. In the current study, we describe the isolation and characterization of a *Rb–/–* prostate epithelial cell line derived from rescued prostate *Rb–/–* tissue. Thus, these models are the first to allow for the continuous study of targeted *Rb* deletion in a specific nonchimeric organ and cell lines past E12.5 of embryonic development. The Rb–/–PrE cell line also provides an excellent experimental platform with which to investigate, for the first time, the physiological consequences of the specific deletion of *Rb* in an epithelial population.

2. Materials

2.1. Tissue Rescue

1. Dissecting instruments (all from Fine Scientific Tools).
2. Large scissors 14054-13.
3. Small scissors 14060-09.
4. Vanna scissors 15100-09.
5. Large forceps 11021-12.
6. Medium forceps 11027-12.
7. No. 3 forceps 11231-30.
8. No. 5 forceps 11252-30.
9. Dissecting scope and light source.
10. 100- and 30-mm Petri dishes—bacteriological dishes are fine for this. They are cheaper than tissue-culture coated plates.
11. Microconcavity slides—These are an off-catalog item from Fisher NC 9583502.
12. Hanks Balanced Salt Solution (HBSS).
13. Syringes Tuberculin type with attached needles (Beckton-Dickenson 309625). Alternatively, 1-mL syringes and 25-gage needles.
14. Sterile Pasteur pipets.
15. Bunsen burner.
16. Blue (1 mL) pipet tips.
17. Calcium/magnesium-free HBSS.
18. Silastic tubing (Fisher Scientific 11-18915G). Now marketed as “laboratory tubing.”
19. Anesthetic: Avertin for Mouse Anesthesia
    Stock Solution: 25 g 2-, 2-, 2-Tribromoethanol (Aldrich T4, 840-2), 15.5 mL tert-Amyl alcohol (Aldrich 24, 048-6).

To prepare avertin stock, mix and warm to 40ºC to dissolve solid. Do not heat. Tribromoethanol is light sensitive, so cover with foil. When completely dissolved wrap in foil and store at 4ºC. If the tribromoethanol recrystallizes, warm again to redissolve. Stock is good for many months.

To make final solution, mix 19.75 mL HBSS with 0.25 mL of avertin stock (you can also use any growth medium, but phenol (ϕ) red is needed to monitor pH). The mixture will have to be gently warmed and stirred to dissolve the stock (45ºC in a water bath is fine). Do not heat excessively as the alcohols will vaporize, and the mixture will not work. Heating should be the minimum required to achieve solution. If the mixture becomes acid (yellow), it has been heated too much and should be discarded. When dissolved, store at 4ºC. This working stock is good for up to 14 d.

To use, inject intraperitoneally. Dosage is 0.02 mL/g body weight (a 25 g mouse gets 0.5 mL, a 30-g mouse gets 0.6 mL). Mice should go down in 2–3 min and will remain asleep for 30–40 min. Check for response to paw squeezing. Individual responses to anesthetic agents do vary slightly, if necessary administer extra anesthetic (0.05–0.1 mL).

Animal Hosts to receive tissue recombinants.

1. CD1 Nude (Charles River) Ideally, use hosts around 60 d of age. Animals less than 45 d old are rather small for this work.
2. Sterile gauze swabs—(Johnson and Johnson 2318).
3. Betadine—Purdue Frederick.
4. 70% ethanol.
5. Sutures—For preference, #3 silk with a small curved needle. The use of silk provides a visual confirmation that a kidney has been grafted. However, please note that some IACUCs will not approve the use of nonresorbable sutures. (Ethicon Inc.)
6. Wound clipper and clips. (Beckton Dickenson—Clipper 427630, Clips 427631.)
7. Heating pad (Gaymar T/Pump TP-500).

Analgesia: These surgeries are well tolerated and historically analgesia has not been used routinely. However, recently, IACUCs have been insisting upon the use of analgesic agents. For these purposes, the approved protocol is:

1. Drug: Buprenorphine (Reckitt & Colman—NDC 12496-0757-1).
2. Dosage: 0.01–0.05 mg/kg.
3. Route: subcutaneously.
4. Frequency: Once at surgery, additionally as needed.

2.2. Tissue Recombination

1. Pregnant rats: For urogenital sinus dissection.
2. Timed pregnant—plug date is d 0.

3. Rats 18 d gestation. (Outbred strains such as Sprague-Dawley are preferred as these produce larger litters than inbred strains)
4. Agar plates: 1% agar (Difco) 5 mL; 2X DME/H16 3.8 mL; Serum (fetal bovine) 1 mL.
5. Trypsin—Sigma or Difco 1:250 (Sigma T-4799).
6. Make a 10 mg/mL solution in calcium/magnesium-free HBSS.
7. DNase type 1—Sigma (DN-25).

2.3. Primary Cultures

1. RPMI-1640, BioWhittaker.
2. Insulin, transferrin, selenium (ITS) (Collaborative Research).
3. Bovine pituitary extract (BPE) (Sigma).
4. Epidermal growth factor (EGF) (Collaborative Research).
5. Cholera Toxin (Sigma).
6. Fungizone (Gibco).
7. Dexamethasone (Sigma).
8. L-glutamine, penicillin G, and streptomycin (Gibco).
9. G418 neomycin (Gibco).
10. PCR primers (Jackson Laboratories, Bar Harbor, MA).
    a. *Rb* wild-type forward 5'-AAT TGC GGC CGC ATC TGC ATC TTT ATC GC-3' (oIMR025).
    b. *Rb* knockout reverse 5'-GAA GAA CGA GAT CAG CAG-3' (oIMR027).
    c. *Rb* wild-type allele reverse 5'-CCC ATG TTC GGT CCC TAG-3' (oIMR026).

2.4. Characterization of Rb–/–PrE Cells

1. pRb antibody 14001A (PharMingen).
2. Donkey antimouse peroxidase conjugated IgG E974 (Amresco).
3. Protease inhibitors; PMSF, leupeptin, aprotinin, sodium orthovanidate.
4. Hoechst 33258 dye (Sigma).
5. Androgen receptor antibody sc-816 and estrogen receptor β antibody sc-8974 Santa Cruz.

3. Methods

3.1. Tissue Rescue

1. Heterozygous (*Rb*+/–) male and female mice can be purchased from the Jackson Laboratory (Bar Harbor, ME) and mated. At 12 d of gestation (plug day denoted as d 0), mothers are sacrificed and fetuses removed and placed under a dissecting microscope where the pelvic visceral rudiments will be removed. This mass of tissue will include the cloaca, and other adjacent organs. This tissue is then grafted beneath the renal capsule of intact male athymic mouse hosts ***(34)*.**
2. Surgery to the renal capsule is somewhat demanding to learn, but is extremely efficient in terms of graft success. There are two sources of training in the subrenal capsule grafting method that may be of interest. The first is the NIH mammary gland

website, *see* http://mammary.nih.gov/tools/mousework/Cunha001/index.html. The second source of information is a DVD of surgical techniques that resulted from a training course entitled "Techniques in Modeling Human Prostate Cancer in Mice," held at The Jackson Laboratory, Bar Harbor, ME, and supported by the NCI Mouse Models of Human Cancer Consortium. This DVD is available from The Jackson Laboratory.

3. The status of the *Rb* gene in the fetuses will be determined by PCR (*see* specific protocol in **Subheading 3.4.,** step 1). After 1 mo of growth, the tissue masses are removed from the renal capsule of the nude mouse hosts and the various structures teased apart under a dissecting microscope. The tissues that develop from these grafts include rectum, prostate, and urinary bladder, as well as other closely associated organs, such as seminal vesicles and genital tubercle. The two major glandular structures found are the seminal vesicles and prostate that are easily distinguished by their characteristic structures and by the color of the secretions that they contain. Prostatic ductal structures can be identified grossly within grafts by their glandular features (*see* **Fig. 1**). Some of this tissue can then be fixed for further immunohistochemical analysis or dissected into small ductal segments for recombination with rat urogenital mesenchyme (rUGM).
4. Although morphologically distinct, the prostatic phenotype of tissues within pelvic visceral grafts should be confirmed by histological and immunohistochemical staining. Tissues can be fixed in 10% formalin overnight, embedded in paraffin, and sectioned on a microtome. Tissue sections can be deparaffinized in Histoclear (National Diagnostic, Atlanta, GA) and hydrated in graded alcoholic solutions and distilled water. Endogenous peroxidase activity should be blocked with 0.5% hydrogen peroxide in methanol for 30 min followed by washing in phosphate-buffered saline (PBS) pH 7.4. Normal goat serum is usually applied to the sections for 30 min to bind nonspecific sites. The sections were then incubated with the primary antibodies overnight at 4ºC or with nonimmune mouse IgG.
5. To confirm the histological lineage of the grafts, a number of tissue- and species-specific antigens can be examined by immunohistochemistry. For prostate grafts, antibodies that recognize the mouse dorsolateral prostate secretory protein (mDLP) and mouse seminal vesicle secretory protein can be used at 1:1500 and 1:5000, respectively ***(35)***, will distinguish between these two tissue components. The employment of antiandrogen-receptor antibody (PA1-111A, 1:100 Affinity BioReagents, Golden, CO) is also useful in determining prostate specific lineage. Monoclonal antibodies specific for mouse cytokeratin 14 and cytokeratin 8 can be used to distinguish basal epithelial cells, which should stain positive for cytokeratin 14 (*see* **Fig. 2a**), whereas the luminal epithelium should express cytokeratin 8 (*see* **Fig. 2b**). Mouse anti-PCNA monoclonal antibody (PC-10, 1:200, PharMingen, San Diego, CA) can also be used to determine the epithelial proliferation rate in *Rb*–/– tissues, which has previously been shown to be higher in the knockout cells ***(33)***. Using the antismooth muscle α-actin monoclonal antibody (A-2547, 1:500, Sigma, St. Louis, MO) and the anti-E-cadherin monoclonal antibody (C20820, 1:200, Transduction Laboratories, San Diego, CA), can be useful in determining if the recombined grafts

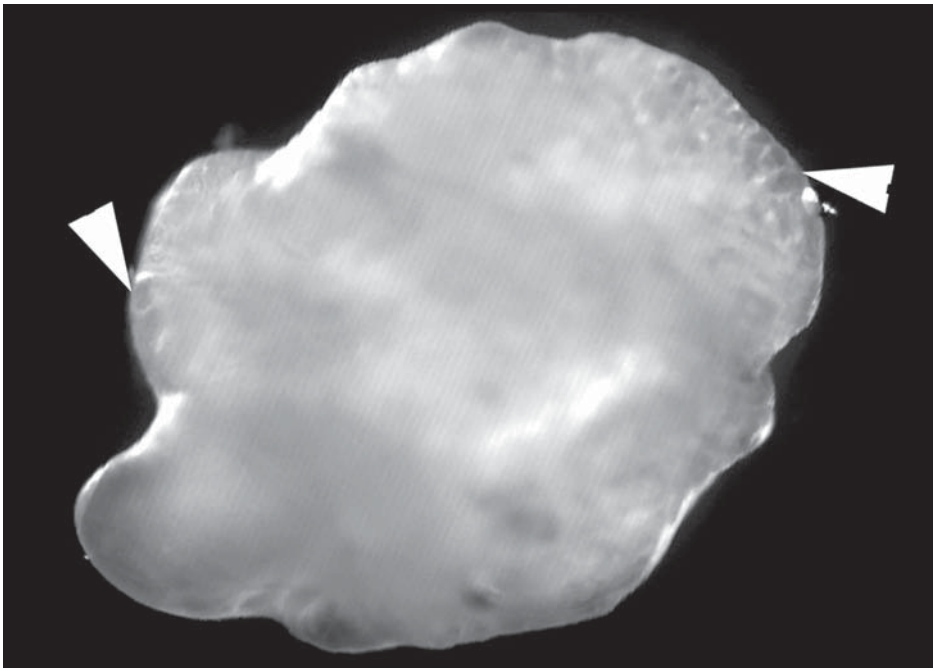

Fig. 1. *Rb–/–* prostatic tissue following rescue and 28 d of growth in an athymic male host. Gross appearance of the *Rb–/–* graft shows a mass of tissue that includes prostate ductal structures (arrowheads).

have achieved normal differentiation and histological architecture. E-cadherin should be observed as strong membranous staining along adjacent epithelial cells (*see* **Fig. 2c**). Actin-positive smooth muscle cells (*see* **Fig. 2d**) should also surround the epithelial ducts and exhibit an intimate association with the epithelial basement membrane. Following all primary antibody incubations, the sections should be washed carefully and then subjected to a secondary incubation in biotinylated goat antimouse immunoglobulin (diluted with PBS at 1:200, Sigma, St. Louis, MO).

6. After incubation with the secondary antibody, sections are then washed in PBS (three 10-min washes), and incubated with avidin-biotin complex for 30 min at room tem-perature. After the last PBS wash, the sections should be developed for about 1–5 min using 3, 3-diaminobenzidine (DAB) in PBS and 0.03% H_2O_2. Sections can then be counterstained with hematoxylin, and dehydrated in alcohol. Control sections can be processed in parallel with mouse nonimmune IgG at the same concentration as the primary antibodies.

3.2. Tissue Recombination

1. UGM is prepared from 18-d embryonic Sprague-Dawley rat fetuses (plug date denoted as d 0). For this purpose, urogenital sinuses should be dissected from fetuses and

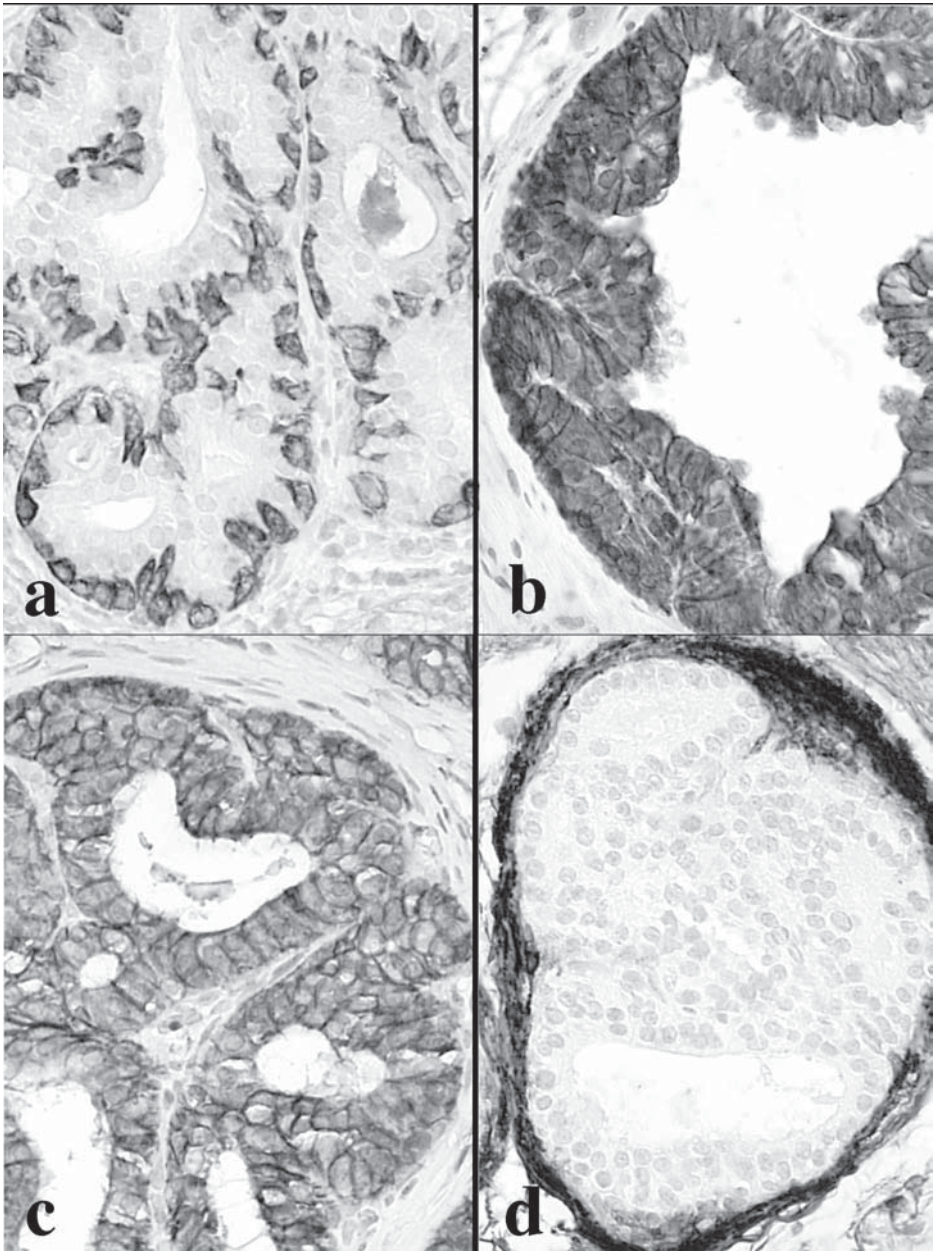

Fig. 2. Rescued *Rb*–/– tissues retains the expression of cell specific antigens. When microdissected and stained these glandular structures express cytokeratin 14 (**a**), cytokeratin 8 (**b**), E-cadherin (**c**) and muscle a-actin (**d**).

separated into epithelial and mesenchymal components following tryptic digestion and mechanical separation. The dissection of the embryonic urogenital sinus is too lengthy to be discussed in detail here. The specific protocol and diagrammatic depiction of the dissection including the location of cuts and the appearance of the various products are illustrated in **ref.** ***34***.

2. Following dissection, the urogenital sinuses should be submerged in a 10 mg/mL solution of 1:250 trypsin in calcium/magnesium-free Hanks solution for approx 75 min either on ice or at 4ºC. Tryptic digestion is then terminated by washing the sinuses three times in medium containing 10% FBS. The epithelial and mesenchymal tissue layers are separated mechanically using no. 5 forceps and a hypodermic needle.
3. Tissue recombinants are prepared by placing *Rb*+/+ and *Rb*–/– prostatic ductal segments (cut into small 200–500 μm pieces) on top of rUGM in dishes containing nutrient agar: 1% agar (Difco), 2X DME/H16, and FBS. Details for this part of the procedure can be found in **ref.** ***36***.
4. After 24 h, the tissue recombinants were grafted underneath the renal capsule of intact male athymic mouse hosts. Following 1 mo of growth, the hosts were sacrificed and the grafts harvested. Pieces of graft were fixed for immunohistochemical characterization. The remainder of the grafts can be used as a source material for the generation of cell cultures. The tissue recombination protocol is shown schematically in **Fig. 3**.

3.3. Primary Isolation and Establishment of Rb*-/- Epithelial Cells*

1. To begin the isolation of *Rb*–/– prostate epithelial cells, recombined prostate grafts were excised and cut into small (approx 1 mm^3) pieces. A portion of each excised graft should be fixed in formalin for histological examination to confirm prostatic phenotype. The remaining samples will be utilized in the preparation of primary epithelial cultures.
2. The tissue should be minced further with a scalpel and forceps and plated onto tissue-culture plastic (Falcon dishes) or on collagen substrate in a minimal volume of medium to allow for attachment of cells and tissue to the matrix.
3. The culture media should consist of either DMEM (#12-604F BioWhittaker) or RPMI-1640 (#12-702F BioWhittaker). Media should be supplemented with ITS ([5 μg/mL] insulin, [5 μg/mL] transferrin, [5 ng/mL] selenium, #40351 Collaborative Research), BPE ([10 μg/mL] bovine pituitary extract, #P1167 Sigma), EGF ([10 μg/mL] Epidermal Growth Factor, #40001 Collaborative Research), Cholera Toxin ([0.01 μg/mL to 1.0 μg/mL], #C-8052 Sigma), amphotericin B ([250 μg/mL], Fungizone #15295-017 Gibco), Dexamethasone ([5 μ*M*], #D-2915 Sigma), [200 m*M*] L-glutamine, 100 U/mL penicilin G, and 100 U/mL streptomycin (#25030-081, #15140-148, respectively, Gibco). This formulation supports the growth of epithelial cells while retarding the growth of fibroblast cells.
4. Approximately 2 wk after plating, individual cells will be present radiating out from the tissue pieces. At this point the cells can be removed from the cultures and neomycin selection initiated. At the start, the cells should be selected with 100 μg/mL

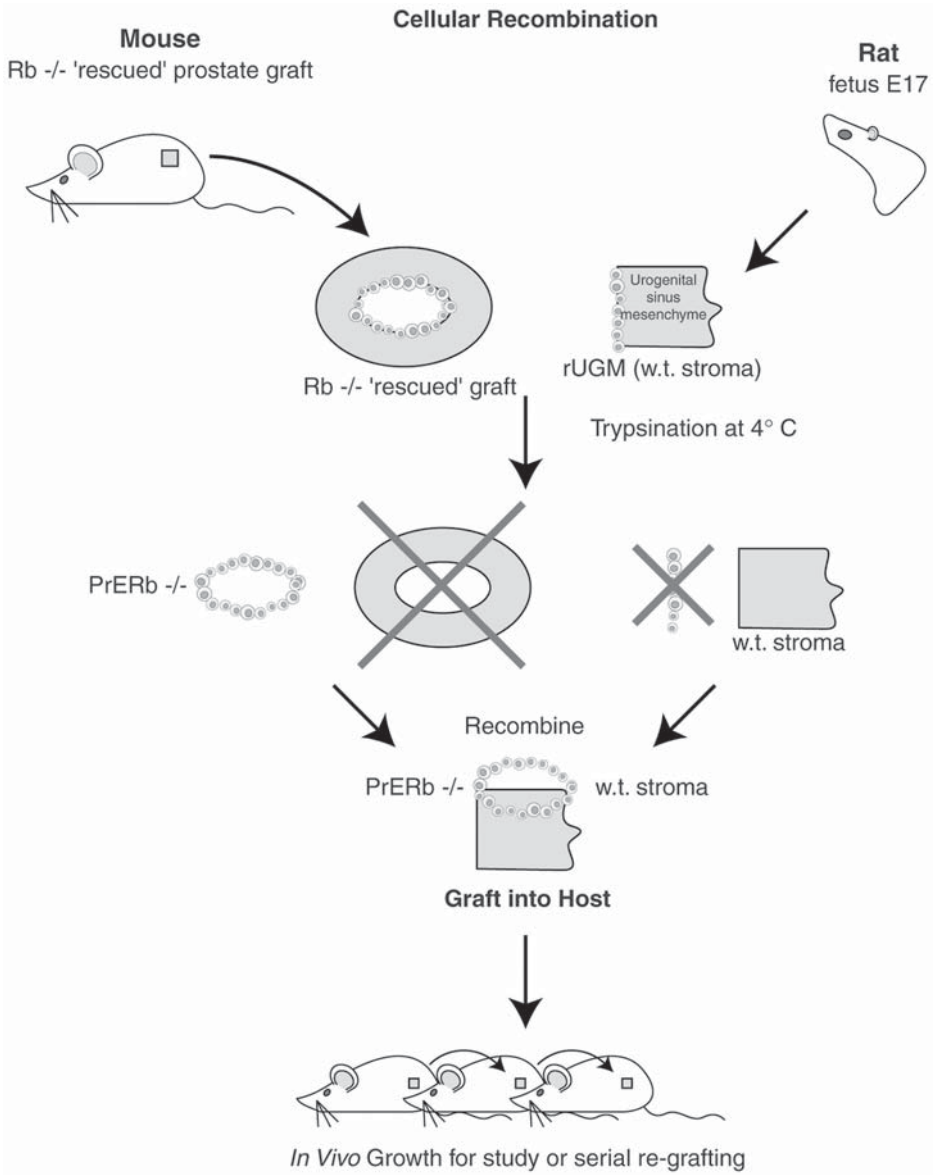

Fig. 3. Schematic of cellular recombination using rUGM and *Rb*–/– prostate epithelium. *Rb*–/– epithelium (*Rb*–/–PrE) isolated from mouse *Rb*–/– rescued prostate tissue are recombined with wild-type rat urogenital sinus mesenchyme (wt stroma) derived from 18-d embryonic rats and grafted beneath the renal capsule of male nude mouse hosts.

G418 (Gibco) and the concentration gradually increased to 200 µg/mL. However the optimal concentration for selection may need to be determined empirically.

5. When large areas of epithelium become established the cells can then be passaged 1:3 by trypsinization and plated into 100-mm dishes under the same neomycin selective pressure (200 µg/mL). At passage 4, the cultures should be completely void of fibroblast cells. At passage 5, 2.5% FBS can be added to the culture medium in addition to the aforementioned growth factors and supplements. At passage 10, the cultures can be weaned off all growth factors and switched to RPMI-1640 supplemented with 5% FBS and 200 µg/mL G418. Cultures can be maintained by splitting once per week at a 1:6 dilution.

3.4. Characterization of Rb*-/- Cell Lines*

1. The genetic status of wild-type or *Rb*–/– grafts and cell lines should be determined by PCR. Preparations of control DNA are commercially available from the Jackson Laboratory and DNA from *Rb*–/– cell lines or tissues can be extracted following standard DNA isolation protocols. Confirmation of the *Rb*–/– genotype during cell isolation or in vivo studies is necessary to assure that the correct cells are employed during the various stages of the experimental protocol. Since the *Rb* gene was disrupted by insertion of the neomycin selection cassette, its presence is indicative of the *Rb*–/– mutation. To distinguish the *Rb*–/– mutation in cultured cells and tissue, the following DNA primers should be used in PCR genotyping. The reverse neomycin-specific primer (oIMR027) binds to its respective sequence in the neomycin resistance cassette and the forward wild-type *Rb* primer (oIMR025) binds to its respective sequence in the remaining *Rb* gene. These primers will amplify a 420 bp product from the mutant *Rb*–/– alleles on a 2.0% agarose gel. The same (oIMR025) primer will be used with another reverse primer (oIMR026) specific for wild-type *Rb* that detects wild-type *Rb* sequence. This primer set will amplify a slightly smaller 400 bp product from the wild-type *Rb*+/+ alleles on a 2.0% agarose gel. These primers are also commercially available from the Jackson Laboratory. As shown in **Fig. 4**, PCRs employing these primer sets reveal the larger 420 bp product from DNA isolated from *Rb*–/– cells compared to the 400 bp wild-type PCR product or the mixed PCR products if heterozygous cells are used.
2. Although PCR genotyping should give a clear indication as to the genetic status of *Rb*–/– cells, it is prudent to confirm the loss of *Rb* by examining the expression of the *Rb* gene product, pRb 110 kDa. The pRb-specific antibody 14001A (PharMingen, Torre Pines, CA) is a monoclonal antibody that recognizes both the hyper- and hypophosphorylated forms of *Rb* and works well for Western blot analysis. The donkey antimouse peroxidase conjugated IgG E974 (Amresco) was used as a secondary antibody for the anti-Rb primary. For protein analysis, cultured cells can be lysed on ice in 300 µL of 50 m*M* Tris-HCl, pH 7.5, 120 m*M* NaCl, 0.5% Nonidet P-40, 1 m*M* EGTA, and protease inhibitors were added fresh: 40 µ*M* PMSF, 5 µg/mL leupeptin, 50 µg/mL aprotinin, 200 µ*M* sodium orthovanidate. Following centrifugation at 15,000*g* for 5 min in a Eppendorf-style centrifuge, the supernatants were collected, quantitated using a Bradford microtiter assay, and prepared in a reducing

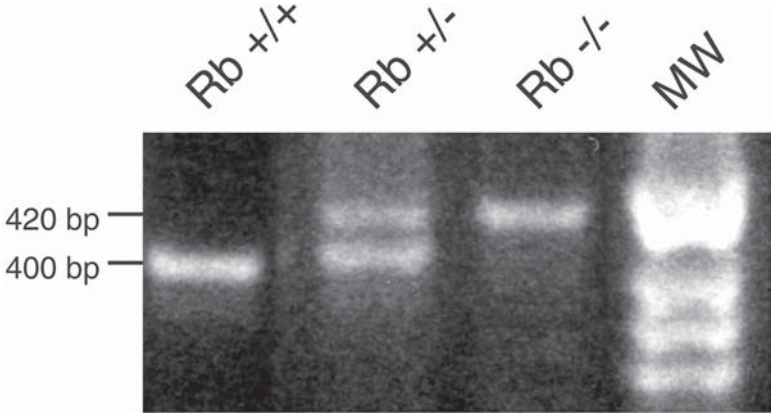

Fig. 4. Verification of *Rb*–/–PrE genotype. Primer sets 025-026 (*Rb*+/+) and 025-027 (*Rb*–/–) amplified only wild-type (wt) alleles (400 bp) from wild-type control cells (*Rb*+/+). The same primer sets amplified wild-type (400 bp) and mutant alleles (420 bp) from heterozygous control cells (*Rb*-/+). Only the mutant allele (420 bp) was amplified with these primer sets from *Rb*–/–PrE cells (*Rb*–/–).

protein sample-loading buffer for electrophoresis. All proteins will then be separated on 6% or Tris/Glycine precast NOVEX gels and analyzed using the ECL (Amersham) detection system. The resulting Western analysis of *Rb*–/– prostate epithelial cells should reveal complete loss of pRb protein expression confirming the *Rb*–/– genotype. As a positive control for the pRb protein and antibody, it is recommended to run the identical amount of lysate from wildtype cells.

3. The use of cellular recombination, employing neomycin-sensitive wild-type rUGM and neomycin-resistant *Rb*–/– mouse epithelium should allow for the specific selection of *Rb*–/– from the wild-type rat stroma or contaminating rat epithelium, however, the absence of rat cells in these cultures should be confirmed by Hoechst 33258 staining. Hoechst staining is widely accepted as a rapid and highly specific method to distinguish mouse cells from contaminating rat cells. Fixed cells should be stained with Hoechst 33258 dye (Sigma, St. Louis, MO) at 5 µg/mL for 1 min at room temperature. Following staining, cultures will be washed three times in PBS, wet mounted, and examined microscopically to confirm that the cells are of mouse origin. Following staining, cells can be examined and photographed using a fluorescent microscope. Examples of Hoechst-stained *Rb*–/– murine and rat epithelium are shown in **Fig. 5**. Microscopic examination should reveal punctate nuclear patterns in the *Rb*–/– cultures characteristic of mouse cells (*see* **Fig. 5A**), while control rat epithelial cells exhibited a homogeneous, nonpunctate staining pattern (*see* **Fig. 5B**).
4. The pRb protein is thought to regulate a postmitotic state required for cellular differentiation and tissue development. However, our initial examination of *Rb*–/–

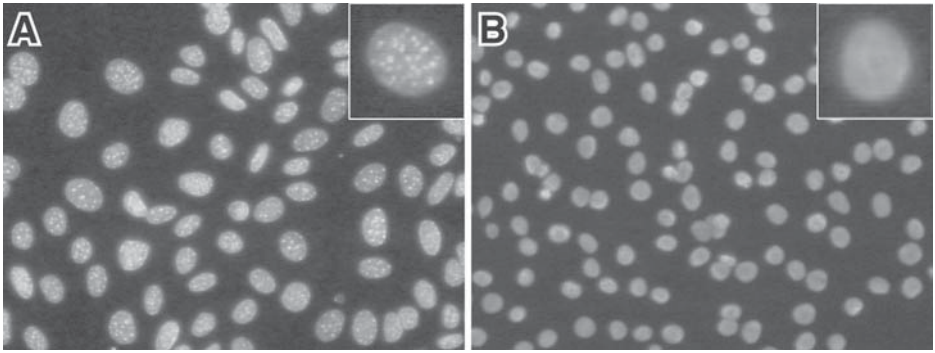

Fig. 5. In vitro characterization of the *Rb–/–*PrE cell line. The punctate staining pattern of the Hoechst 33258 stain is evident in mouse *Rb–/–*PrE cells (**A**), although absent in control rat epithelium (**B**).

prostate epithelial cells revealed normal epithelial morphology. Several steps should be taken at this point in the culturing of *Rb–/–* cells to determine their state of differentiation. Initially, simple examination of cellular morphology can reveal much about the cell line in question. Does the phenotype reflect a large, flattened morphology indicative of basal cells or is the phenotype consistent with spindle-shaped luminal cells? The exact delineation of epithelial lineage will require a molecular assessment of the expression of both epithelial and tissue-specific markers. In the case of *Rb–/–* prostate epithelium, a cytokeratin profile can be performed using antibodies directed against cytokeratins 14 and 8 as described for fixed tissue in **Subheading 3**. Immunohistochemistry is an efficient application of these antibodies in cultured cells, allowing for both the evaluation of specific cytokeratins coupled to morphologic profile. As shown in **Fig. 6A,B**, the morphology of the *Rb–/–* cultures suggests a mixed epithelial phenotype. Using cytokeratin-specific antibodies in conjunction with cell morphology revealed large, flattened cells that were strongly positive for cytokeratin 14 expression, suggestive of a basal epithelial phenotype (*see* **Fig. 6C**). The predominant spindle-shaped cells, expressing cytokeratin 8 are likely of luminal epithelium origin (*see* **Fig. 6D**). Expression of prostate-specific antigens, such as the androgen receptor (sc-816 Santa Cruz, Santa Cruz, CA), estrogen receptor β (sc-8974 Santa Cruz) or mouse dorsal lateral protein ***(35)*** can also be examined by Western blot analysis (*see* protocol above). Western blotting of *Rb–/–* cultures and tissues are useful when quantitative analysis of proteins is desired.

5. All indications from experiments examining differentiation markers of cultured *Rb–/–* prostate epithelial cells demonstrated that these cells have not undergone any dramatic dedifferentiation as a result of *Rb* loss. Although somewhat involved, examining the ability of *Rb–/–* cells to differentiate and develop into normal prostate tissue, following multiple passages in culture, is the best confirmatory test to determine the role of *Rb* in cellular differentiation and tissue morphogenesis. We

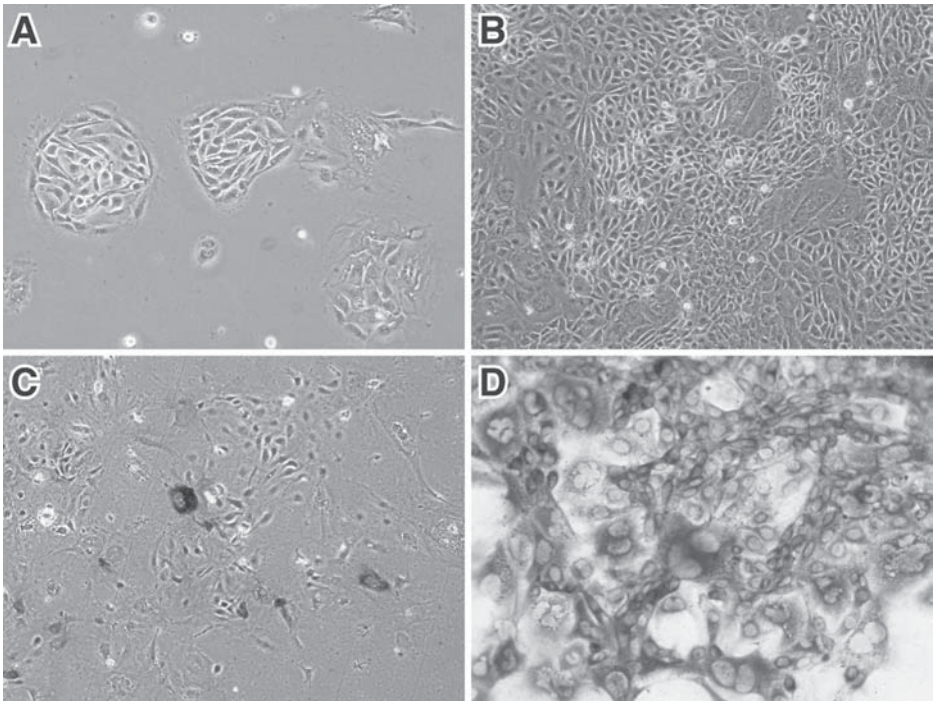

Fig. 6. In vitro characterization of the Rb–/–PrE cell line. Phase-contrast microscopy of subconfluent (**A**) and confluent (**B**) cultures of Rb–/–PrE. Rb–/–PrE cultures immunostained with antibodies against cytokeratin 14 (**C**) and cytokeratin 8 (**D**).

have determined that Rb–/–PrE cells could recapitulate prostate tissue in vivo. These same experiments can be achieved by combining Rb–/–PrE (passage 15) with rUGM, grafted to intact male athymic mouse hosts for 8 wk at which time the *Rb*–/– genotype can be confirmed by PCR. The prostate grafts are microdissected and used in a second round of recombination and regrafting in a male athymic host to produce a second-generation graft. Prostatic histodifferentiation of these serial regrafts can then be compared to wild-type grafts. As shown in **Fig. 7**, microscopic examination revealed normal prostate glandular morphology. In addition the expression of prostate-specific antigens, such as the androgen receptor and mouse dorsal lateral protein can be examined to confirm the murine prostate lineage of the grafts.

3.5. Summary

Due to the lack of a viable *Rb* knockout, experiments to examine the physiologic function for *Rb* in the prostate gland have not previously been accomplished. The technology outlined in this chapter describes innovative models

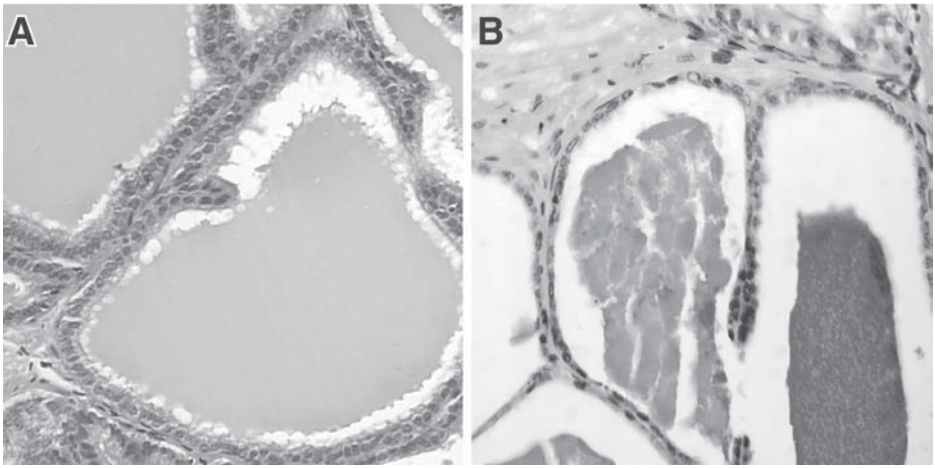

Fig. 7. *Rb*–/–PrE cells recapitulate prostatic histodifferentiation in vivo. Wild-type *Rb*+/+ (**A**) and passage 15 *Rb*–/–PrE cells (**B**) form normal prostate ductal architecture in the tissue recombination.

employing tissue and cellular recombination to analyze homozygous deletion of the *Rb* gene in prostate tissue grafts and cell lines. These models are the first to allow for the continuous study of targeted *Rb* deletion in specific non-chimeric organs past E12.5 of embryonic development and provide excellent experimental platforms with which to investigate the physiological consequences of *Rb* deletion on an epithelial population. Although more conventional methods using transforming oncogenes have undoubtedly been useful in *Rb* research, these reagents do not specifically target *Rb* and result in such severe genetic instability that mechanistic interpretation becomes difficult. These models will be used to generate data on many aspects of *Rb* function. They have the potential to reveal novel functions and downstream targets of *Rb* and to elucidate the role of *Rb* in epithelial signaling, differentiation and death. Similar approaches could also be applied to a range of late embryonic or perinatal-lethal gene mutations and knockout mice.

References

1. Cunha, G. R., Hayward, S. W., Dahiya, R., and Foster, B. A. (1996) Smooth muscle-epithelial interactions in normal and neoplastic prostatic development. *Acta Anatomica* **155,** 63–72.
2. Hayward, S. W., Cunha, G. R., and Dahiya, R. (1996) Normal development and carcinogenesis of the prostate: A unifying hypothesis. *Ann. NY Acad. Sci.* **784,** 50–62.
3. Hayward, S. W., Rosen, M. A., and Cunha, G. R. (1997) Stromal-epithelial interactions in normal and neoplastic prostate. *Br. J. Urol.* **79(Suppl. 2),** 18–26.

4. Cooke, D. B., Quarmby, V. E., Mickey, D. D., Isaacs, J. T., and French, F. S. (1988) Oncogene expression in prostate cancer: Dunning R3327 rat dorsal prostatic adenocarcinoma system. *Prostate* **13,** 263–272.
5. Thompson, T. C., Kadmon, D., Timme, T. L., Merz, V. W., Egawa, S., Krebs, T., et al. (1991) Experimental oncogene induced prostate cancer, in *Prostate Cancer: Cell and Molecular Mechanisms in Diagnosis and Treatment* (Isaacs, J. T., ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp. 55–72.
6. Peehl, D. M. (1993) Oncogenes in prostate cancer. *Cancer* **71,** 1159–1164.
7. Wang, Y. Z. and Wong, Y. C. (1997) Oncogenes and tumor suppressor genes in prostate cancer: a review. *Urol. Oncol.* **3,** 41–46.
8. Sarkar, F. H., Sakr, W., Li, Y. W., Macoska, J., Ball, D. E., and Crissman, J. D. (1992) Analysis of retinoblastoma (Rb) gene deletion in human prostatic carcinomas. *Prostate* **21,** 145–152.
9. Isaacs, W. B., Bova, G. S., Morton, R. A., Bussemakers, M. J., Brooks, J. D., and Ewing, C. M. (1994) Genetic alterations in prostate cancer. *Cold Spring Harb. Symp. Quant. Biol.* **59,** 653–659.
10. Fearon, E. R. and Vogelstein, B. (1990) A genetic model for colorectal tumorigenesis. *Cell* **61,** 759–767.
11. Martin, K. J. and Sager, R. (1998) Expression genetics in cancer research, prognosis, and therapy. *Adv. Exp. Med. Biol.* **451,** 1–7.
12. Bookstein, R., Rio, P., Madreperla, S. A., Hong, F., Allred, C., Grizzle, W. E., and Lee, W. H. (1990) Promoter deletion and loss of retinoblastoma gene expression in human prostate carcinoma. *Proc. Natl. Acad. Sci. USA* **87,** 7762–7766.
13. MacGrogan, D. and Bookstein, R. (1997) Tumour suppressor genes in prostate cancer. *Semin. Cancer Biol.* **8,** 11–19.
14. Yeh, S., Miyamoto, H., Nishimura, K., Kang, H., Ludlow, J., Hsiao, P., et al. (1998) Retinoblastoma, a tumor suppressor, is a coactivator for the androgen receptor in human prostate cancer DU145 cells. *Biochem. Biophys. Res. Commun.* **248,** 361–367.
15. Weinberg, R. A. (1995) The retinoblastoma protein and cell cycle control. *Cell* **81,** 323–330.
16. Dyson, N. (1998) The regulation of E2F by pRb-family proteins. *Genes Dev.* **12,** 2245–2262.
17. Dalbagni, G., Cordon-Cardo, C., Reuter, V., and Fair, W. R. (1995) Tumor suppressor gene alterations in bladder carcinoma. Translational correlates to clinical practice. *Surg. Oncol. Clin. N Am.* **4,** 231–240.
18. Trudel, M., Mulligan, L., Cavenee, W., Margolese, R., Cote, J., and Gariepy, G. (1992) Retinoblastoma and p53 gene product expression in breast carcinoma: immunohistochemical analysis and clinicopathologic correlation. *Hum. Pathol.* **23,** 1388–1394.
19. Tamura, G., Maesawa, C., Suzuki, Y., Kashiwaba, M., Ishida, M., Saito, K., and Satodate, R. (1994) Improved detection of loss of heterozygosity at retinoblastoma gene locus in human breast carcinoma. *Pathol. Int.* **44,** 34–38.
20. Berns, E. M., de Klein, A., van Putten, W. L., van Staveren, I. L., Bootsma, A., Klijn, J. G., and Foekens, J. A. (1995) Association between RB-1 gene alterations and factors of favourable prognosis in human breast cancer, without effect on survival. *Int. J. Cancer* **64,** 140–145.

21. Mori, N., Yokota, J., Akiyama, T., Sameshima, Y., Okamoto, A., Mizoguchi, H., et al. (1990) Variable mutations of the RB gene in small-cell lung carcinoma. *Oncogene* **5,** 1713–1717.
22. Reissmann, P. T., Koga, H., Takahashi, R., Figlin, R. A., Holmes, E. C., Piantadosi, S., et al. (1993) Inactivation of the retinoblastoma susceptibility gene in non-small-cell lung cancer. The Lung Cancer Study Group. *Oncogene* **8,** 1913–1919.
23. Linardopoulos, S., Gonos, E. S., and Spandidos, D. A. (1993) Abnormalities of retinoblastoma gene structure in human lung tumors. *Cancer Lett.* **71,** 67–74.
24. Phillips, S. M., Morton, D. G., Lee, S. J., Wallace, D. M., and Neoptolemos, J. P. (1994) Loss of heterozygosity of the retinoblastoma and adenomatous polyposis susceptibility gene loci and in chromosomes 10p, 10q and 16q in human prostate cancer. *Br. J. Urol.* **73,** 390–395.
25. Phillips, S. M., Barton, C. M., Lee, S. J., Morton, D. G., Wallace, D. M., Lemoine, N. R., and Neoptolemos, J. P. (1994) Loss of the retinoblastoma susceptibility gene (RB1) is a frequent and early event in prostatic tumorigenesis. *Br. J. Cancer* **70,** 1252–1257.
26. Melamed, J., Einhorn, J. M., and Ittmann, M. M. (1997) Allelic loss on chromosome 13q in human prostate carcinoma. *Clin. Cancer Res.* **3,** 1867–1872.
27. Kubota, Y., Fujinami, K., Uemura, H., Dobashi, Y., Miyamoto, H., Iwasaki, Y., et al. (1995) Retinoblastoma gene mutations in primary human prostate cancer. *Prostate* **27,** 314–320.
28. Ittmann, M. M. and Wieczorek, R. (1996) Alterations of the retinoblastoma gene in clinically localized, stage B prostate adenocarcinomas. *Hum. Pathol.* **27,** 28–34.
29. Brooks, J. D., Bova, G. S., and Isaacs, W. B. (1995) Allelic loss of the retinoblastoma gene in primary human prostatic adenocarcinomas. *Prostate* **26,** 35–39.
30. Bookstein, R. (1994) Tumor suppressor genes in prostatic oncogenesis. *J. Cell. Biochem.* **19(Suppl.),** 217–223.
31. Vesalainen, S. and Lipponen, P. (1995) Expression of retinoblastoma gene (Rb) protein in T12M0 prostatic adenocarcinoma. *J. Cancer Res. Clin. Oncol.* **121,** 429–433.
32. Geradts, J., Hu, S., Lincoln, C., Benedict, W., and Xu, H. (1994) Aberrant RB gene expression in routinely processed, archival tumor tissues determined by three different anti-RB antibodies. *Int. J. Cancer* **58,** 161–167.
33. Wang, Y., Hayward, S., Donjacour, A., Young, P., Jacks, T., Sage, J., et al. (2000) Sex hormone-induced carcinogenesis in Rb-deficient prostate tissue. *Cancer Res.* **60,** 6008–6017.
34. Cunha, G. R. and Donjacour, A. A. (1987) Mesenchymal-epithelial interactions: Technical considerations, in *Assessment of Current Concepts and Approaches to the Study of Prostate Cancer* (Coffey, D. S., Bruchovsky, N., Gardner, W. A., Resnick, M. I., and Karr, J. P., eds.), A.R. Liss, New York, pp. 273–282.
35. Donjacour, A. A., Rosales, A., Higgins, S. J., and Cunha, G. R. (1990) Characterization of antibodies to androgen-dependent secretory proteins of the mouse dorsolateral prostate. *Endocrinology* **126,** 1343–1354.
36. Cunha, G. R., Lung, B., and Kato, K. (1977) Role of the epithelial-stromal interaction during the development and expression of ovary-independent vaginal hyperplasia. *Dev. Biol.* **56,** 52–67.

3

Signal Transduction Study Using Gene-Targeted Embryonic Stem Cells

Hideki Kawasome, Takashi Hamazaki, Tetsuo Minamino, and Naohiro Terada

Abstract

Gene targeting is one of the most powerful tools to define the role of signaling molecules in animal development and disease etiology. By using this technique, nearly 1000 knockout mice have been produced over the last two decades. Generating knockout mice, however, is a time-consuming procedure. Also, an unexpected embryonic lethality sometimes prevents us from examining the function of the gene in specific tissues. Here, we describe a convenient method to directly disrupt genes at both alleles in murine embryonic stem (ES) cells. These homozygous knockout ES cells have been shown useful to determine the role of the genes in the mediation of various cellular activities such as proliferation, differentiation, apoptosis, survival, transformation, and so on. Furthermore, with the recent advance of in vitro differentiation techniques, it is now feasible to rapidly determine the role of specific molecules in particular tissues.

Key Words: Gene targeting; embryonic stem cells; homologous recombination; homozygous knockout; heterozygous knockout; in vitro differentiation; signal transduction; p70 S6 kinase; mitogen activated protein kinase.

1. Introduction

1.1. Homozygously Gene-Targeted Embryonic Stem Cells

Embryonic stem (ES) cells are continuously growing stem cell lines of embryonic origin first isolated from the inner cell mass of blastocysts ***(1,2)***. The distinguishing features of ES cells in mice are their capacity to be indefinitely

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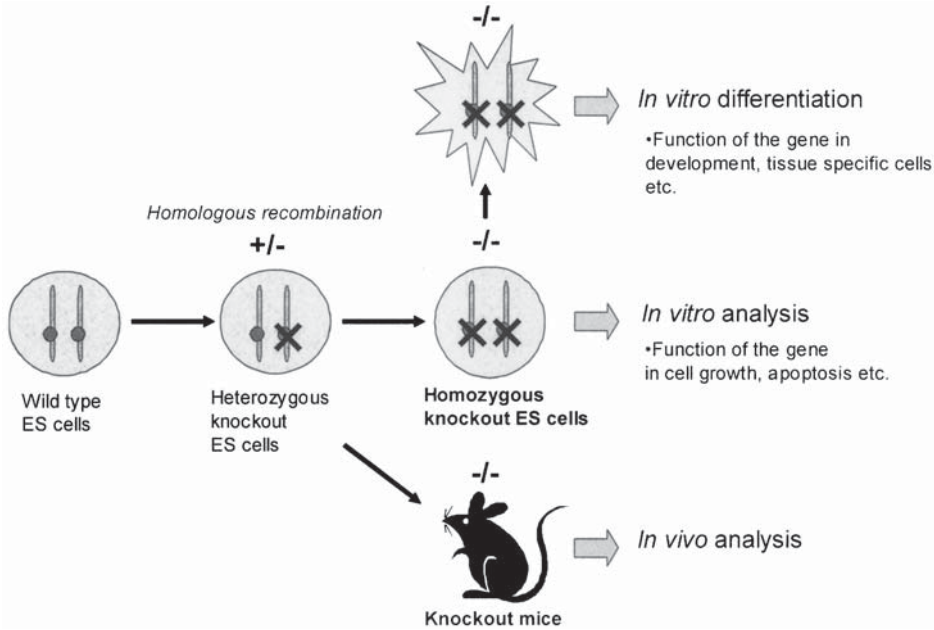

Fig. 1. Overview of the gene-targeting strategy. Generation of homozygous knockout ES cells allows us to analyze the role of the targeted molecules in vitro without making knockout mice. Furthermore, we are able to investigate the function of the genes in specific cell types rapidly in combination with in vitro differentiation techniques of ES cells.

maintained in an undifferentiated state in culture and their potential to develop into every cell type, including germ line, when they are injected into mouse blastocysts ***(3)***. Furthermore, chromosomes of ES cells are fairly stable, and homologous recombination commonly occurs between genome and introduced compatible sequences ***(4)***. By using these unique features of ES cells, gene-targeting techniques have been developed, and a great number of knockout mice have been produced in the last two decades. These gene disruption studies are constructing persuaded frameworks of numerous signaling molecules, which are involved in the mediation of cellular activities such as proliferation, differentiation, apoptosis, survival, and transformation. Here, we describe a convenient method to directly disrupt genes at both alleles in murine ES cells to determine the role of the targeted genes in ES cells (*see* **Fig. 1**). Using this method, we homozygously disrupted the *p70* S6 kinase ($p70^{s6k}$, *S6K1*) gene in murine embryonic stem cells to determine the role of the kinase in cell growth, protein synthesis, and rapamycin sensitivity ***(5)***.

1.2. In Vitro ES Cell Differentiation

In addition to their pluripotent ability to differentiate in vivo, ES cells can also differentiate into multiple cell lineages in vitro as well. The in vitro differentiation of ES cells is induced by removing the mouse embryonic fibroblast (MEF) feeder layer or leukemia inhibitory factor (LIF) from the ES cell culture, and usually by allowing them to form aggregates in suspension. ES cells aggregate into structures termed embryoid bodies (EB), in which all three germ layers develop and interact with each other. Well-differentiated EBs are composed of multiple differentiated cell types including neuronal, cardiac muscle, hematopoietic, and chondrocytic cells. EBs recapitulate many processes that take place during normal embryonic development ***(6)***. Certain aspects of the kinetics of lineage development observed within EBs show remarkable similarities to those observed in the developing embryo ***(7)***. Further, many researchers have been developing the techniques to isolate a specific cell type from differentiating ES cells in vitro. Combined with the homozygous gene-targeting described earlier and in vitro differentiation techniques, it is now feasible to rapidly determine the role of a specific molecule in specific tissues ***(8–14)***. We demonstrated the usefulness of this in vitro ES differentiation system combined with targeted gene disruption to define complex regulatory events in a cardiac disease model ***(15)***. Using cardiac myocytes derived from MEKK1 null ES cells in vitro ***(16)***, we successfully demonstrated a role of mitogen-activated protein kinases in myocardial injury by oxidative stress. This in vitro method is particularly useful when gene disruption causes embryonic lethality. We were able to analyze the role of SEK1 in late hepatic maturation using this method ***(17)***, despite the embryonic lethality of SEK1 knockout mice.

Finally, these in vitro approaches would be very useful with human ES cells ***(18,19)***, where in vivo knockout study is not allowed.

2. Materials

2.1. Mouse ES Cells and Maintenance (20)

ES cells (R1) were maintained on feeder cells (STO cells or Mouse Embryonic Fibroblast). They were also cultured on gelatinized plates instead of feeder cells for a short period, particularly when we needed pure ES cells without feeder cells for biochemical analysis etc (*see* **Note 1**).

2.1.1. Cells

R1 cells and STO cells were kindly provided by Dr. A. Nagy (Mt. Sinai Hospital, Toronto) and Dr. G. M. Keller (Mt. Sinai School of Medicine, NY). This STO cell line was genetically manipulated and resistant to G418 for antibiotic screening of ES cells.

2.1.2. ES Cell Medium

1. D-MEM high glucose (Gibco, 11995-073), 425 mL.
2. Fetal calf serum (FCS, heat inactivated; check lot for the ability to maintain ES cells undifferentiated), 75 mL (*see* **Note 2**).
3. Monothioglycerol (Sigma, M-6145), 1:10 dilution, buy every 3 mo, 62 µL.
4. Penicillin-streptomycin, liquid (Gibco, 15070-063), 5 mL.
5. LIF (Chemicon, ESG1107), 50 µL.
6. HEPES buffer solution (1 *M*) (Gibco, 15630-106), 12.5 mL.

2.1.3. Trypsin/EDTA

1. Trypsin 1:250 (Sigma T-4799), 1.25 g.
2. 0.5 *M* ethylenediamine tetraacetic acid (EDTA), 1.05 mL.
3. PBS(-), 500 mL (*see* **Note 3**).

2.1.4. Gelatin

1. Add 0.5 g of gelatin (Sigma G1890) in 500 mL PBS(-), autoclave, and keep at 4ºC.
2. Cover the culture plates with gelatin solution for 20 min at room temperature and remove it before plating STO or ES cells.

2.1.5. STO Cell Culture

1. Culture STO cells in ES cell medium without LIF in gelatinized flask.
2. Treat confluent STO cells with 6000–10,000 rads of gamma irradiation before plating ES cells (*see* **Note 4**).

2.1.6. 2X Cell Stock Solution

1. 20% dimethyl sulfoxide (DMSO).
2. 80% FCS.

2.1.7. Prepare ES Cells From a Frozen Stock

1. Thaw cells at 37ºC and wash once with medium.
2. Add 5 mL of medium and pipeting.
3. Transfer cells into T-25 flask with feeder cells and culture at 37ºC, 5% CO_2.
4. Change medium at d 1 or d 2 and passage at d 3.

2.1.8. Passage of ES Cells

1. Discard medium and wash once with 5 mL of PBS(-).
2. Add 0.5 mL of Trypsin/EDTA and sit for 2–3 min at room temperature.
3. Tap the flask to remove cells.
4. Add 5 mL of medium and pipeting.
5. Transfer cells into T-75 flask with feeder cells and add medium to 15 mL.
6. Change medium at d 1 or d 2 and passage at d 3.

2.2. Targeting Vectors

2.2.1. Cloning of Genomic DNA Coding a Target Gene

A genomic DNA coding the gene of interest for constructing a targeting vector is needed. A genomic library from the same strain of mice with the ES cells should be used. Plaque hybridization was performed to get a genomic DNA in this section (*see* **Note 5**).

1. Library: 129SV Mouse Genomic Library in the Lambda FIXII Vector was purchased from Stratagene and screening was performed following manufacture's protocol (Stratagene, La Jolla, CA).
2. Screening:
   a. Prepare 10 plates with about 50,000 plaques of genomic library per 150-mm plate.
   b. Refrigerate the plates for 2 h at 4ºC.
   c. Put nylon filters on the plates to lift plaques for 2 min, denature in 1.5 *M* NaCl, 0.5 *M* NaOH for 2 min, neutralize in 1.5 *M* NaCl, 0.5 *M* Tris-HCl pH 8.0 for 5 min, and rinse in 0.2 *M* Tris-HCl pH 7.5, 2X SSC (20X SSC: 3 *M* NaCl, 0.3 *M* sodium citrate, pH 7.0).
   d. Crosslink DNA to the filters for 30 s using auto-crosslink setting on the Stratalinker UV crosslinker (Stratagene).
   e. Prehybridize the filters in 2X PIPES, 50% formamide, 0.5% sodium dodecyl sulfate (SDS), and 100 µg/mL of denatured and sonicated salmon sperm DNA for 2 h at 42ºC.
   f. Label the probe and hybridize to the filters in 2X PIPES, 50% formamide, 0.5% sodium dodecyl sulfate (SDS), and 100 µg/mL of denatured and sonicated salmon sperm DNA overnight at 42ºC.
   g. Wash the filters in 0.1X SSC, 0.1% SDS at 60ºC, and expose to X-ray film overnight at −70ºC.

2.2.2. Construction of a Targeting Vector

1. Analysis of Genomic DNA
   a. Digest a cloned genomic DNA with several restriction enzymes and prepare restriction map.
   b. Hybridize cDNA probe to each digested fragment for determining the position of exons. Confirm the exons by sequencing.
2. Construction of a targeting vector ***(21)***.

Figure 2 illustrates a targeting vector we made when we disrupted *p70* S6 kinase gene ***(5)***. A neomycin resistance gene was inserted to disrupt one exon, shown in Fig. 2B. HSV thymidine kinase (HSVtk) gene was inserted for negative selection. When homologous recombination has occurred (*see* **Fig. 2C**), the cells become resistant to G418 and Gancyclovir. Arrowheads indicate primers for screening by polymerase chain reaction (PCR). If the cells have a knockout allele, the *Neo1* and *PS1* primer set can amplify a DNA fragment. If the cells

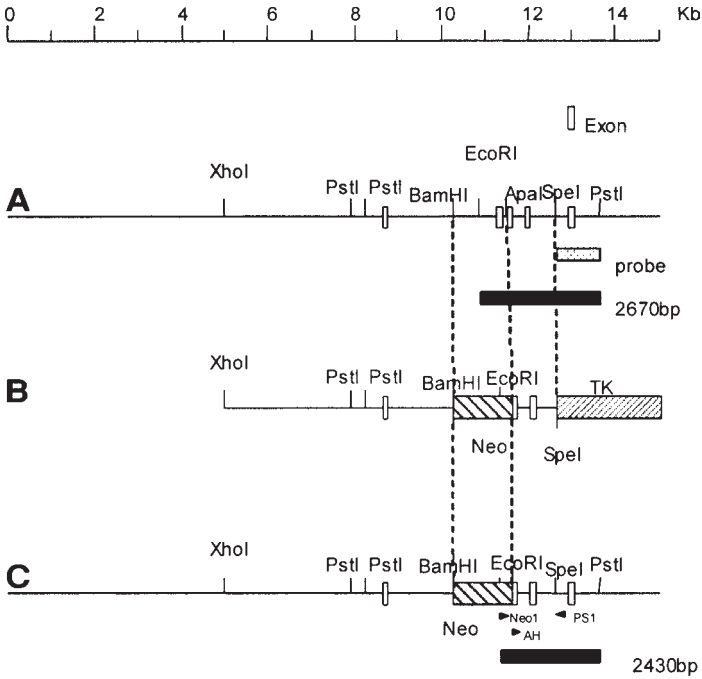

Fig. 2. Structure of the genome coding *p70* S6 kinase and targeting vector. (**A**) Genome encoding *p70* S6 kinase. (**B**) Targeting vector. (**C**) Expected structure of the genome after homologous recombination.

have a wild-type allele, the *AH* and *PS1* primer set can amplify a DNA fragment. The genotype can be determined using both sets of primers. Note that the position of the primer *PS1* is out of the targeting vector. This is important for detecting a homologous recombination from random inserting. We designed the vector having one short arm for PCR screening. The probe in **Fig. 2A** was used for Southern blot. After the digestion with *Eco*RI and *Pst*I, the wild-type allele shows 2670 bp band and the knockout allele shows 2430 bp.

3. Methods

3.1. Heterozygous Gene Targeting

3.1.1. Transfection of a Targeting Vector

1. Trypsinize ES cells to single cells, add medium, and incubate on culture dishes for 15 min to let feeder cells attach to the dishes.
2. Transfer the suspended cells to new tubes, wash, and resuspend in PBS(-).
3. Mix 0.8 mL of the cell suspension (1×10^7 cells) with 30 µg of linearized vector DNA, and transfer into an electroporation cuvet.

4. Deliver the electric pulse by BioRad Gene Pulser at 230 V, 500 µF. Place the cuvet at room temperature for 20 min. (Try several different conditions to obtain maximum efficiency. We performed 180, 230, 240, 250, and 300 V and obtained the most number of colonies with 230 V.)
5. Plate the cells to three 100-mm dishes and incubate at 37ºC, 5% CO_2.
6. Add G418 at 500 µg/mL and gancyclovir at 4 µ*M* for drug selection in 2 d after the electroporation.
7. Culture the cells for about 12 d by changing media every other day.

3.1.2. Making a Stock of Each Colony and DNA Extraction for Analysis

1. Pick up the G418 and gancyclovir-resistant colonies with a pipet (P-20 tip with filter) under an inverted microscope. Transfer a colony into a 96-well round-bottom plate with 50 µL/well of trypsin/EDTA and incubate for 10 min at 37ºC. Pipet the cells and transfer to a 24-well plate with feeder cells and 1 mL of medium.
2. After the cells have grown to 50% confluence, wash once with PBS(-) and incubate with 100 µL of trypsin/EDTA for 5 min at 37ºC.
3. Add 750 µL of medium and pipet gently for breaking the clumps.
4. Transfer 250 µL of cell suspension to a new gelatin-coated 24-well plate for DNA analysis.
5. Mix remaining 500 µL of cell suspension with 500 µL of 2X cell stock solution. Freeze at –20ºC for 15 min, at –80ºC overnight, and keep in liquid nitrogen afterward.
6. After the cells for DNA analysis have grown to 50% confluence, wash once with PBS(-), and incubate overnight at 37ºC with 500 µL of a lysis buffer containing 10 m*M* Tris-HCl (pH 8.0), 25 m*M* EDTA (pH 8.0), 0.5% SDS, 75 m*M* NaCl, and 100 µg/mL proteinase K.
7. Extract DNA by phenol/chloroform, precipitate in ethanol, and dissolve in 100 µL of water.

3.1.3. Screening by PCR

1. PCR mixture contains 250 n*M* of each primer, *Neo1* or *AH* and *PS*1 (*see* **Fig. 2**), 200 µ*M* of each deoxynucleotide triphosphate, 50 m*M* KCl, 10 m*M* Tris-HCl, pH 8.3, 1.5 m*M* magnesium acetate, 1.25 unit of *Taq* DNA polymerase, and 5 µL of template DNA in total volume of 50 µL.
2. PCR conditions include an initial incubation at 94ºC for 2 min followed by 35 cycles of 1 min at 94ºC, 1 min at 55ºC, 3 min at 72ºC, with a final incubation for 5 min at 72ºC.
3. Analyze PCR products by electrophoresis in agarose gel.

3.2. Homozygous Gene Targeting

*3.2.1. Selection of High G418-Resistant ES Cells (see **Note 6**).*

1. Trypsinize single-allele knockout ES cell clones and plate into 100-mm plates with feeder cells at the density of 10^5 cells per plate.

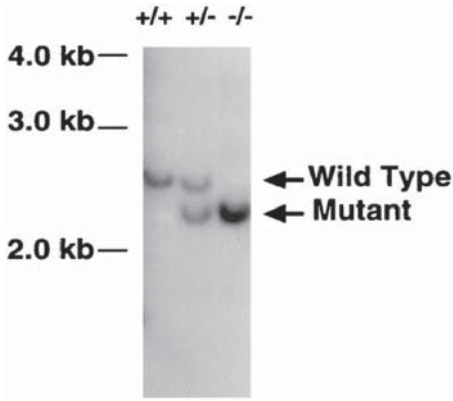

Fig. 3. Southern blot for determining homozygous targeted ES cells.

2. Culture the cells with 3, 6, or 10 mg /mL of G418 for 1 wk, changing media every 2 d.
3. Pick up the G418-resistant colonies and make stock and DNA samples as described earlier.

3.2.2. Screening by Southern Blot ***(22)***

Southern blot was used to detect homozygous knockout cells because R1 cells were cultured on feeder cells (mouse fibroblast), and the PCR method had a risk to detect the wild-type genome of feeder cells.

1. Digest DNA with *Eco*RI and *Pst*I, and separate by 1% agarose electrophoresis.
2. After denaturing and neutralizing DNA, transfer DNA to a nylon membrane in 10X SSC, and fix by ultraviolet irradiation.
3. Hybridize labeled DNA to the membrane in 50% formamide, 6X SSC, 0.5% SDS, and 100 µg/mL salmon sperm DNA at 42ºC overnight.
4. Wash the membrane in 0.1% SDS and 0.1X SSC solution at 60ºC, and take an autoradiograph overnight (*see* **Fig. 3**).

3.3. In Vitro Differentiation

It is now feasible to generate and isolate a variety of tissue-specific cells from differentiating ES cells. The detailed methods are described in *Methods in Molecular Biology*, vol. 185 (2002) ***(23)***. Briefly, the in vitro differentiation of ES cells is basically induced by removing the ES cells from the feeder layer and by removing LIF from the culture medium. When differentiating ES cells were cultured in suspension on Petri dish, ES cells aggregate and form EBs that spontaneously differentiate into various cell types including cardiac myocytes, neuronal cells,

erythrocytes, melanocytes and others ***(6)***. Enrichment and/or isolation of certain types of cells has been achieved, in some cases, by addition of various growth/differentiation factors or chemicals. For example, pure populations of mast cell precursors can be easily obtained from mouse ES cells using interleukin-3 and stem cell factor (*c*-kit ligand) ***(8)***. In other cases, tissue specific-precursors can be sorted using FACS based on expression of specific markers on the cell surface. *Flk*1 positive cells from mouse ES cells were demonstrated to serve as vascular progenitors ***(24)***. Tissue-specific promoter-derived drug selection has been used to purify other cell types including cardiac myocytes and insulin-secreting cells ***(25,26)***. Similarly, tissue-specific promoter-derived GFP expression and subsequent FACS sorting have been used to purify cardiac myocytes ***(27)***, neural precursors ***(28)***, and hepatocytes (Hamazaki et al., submitted). ES cells can also be differentiated into specific lineages by coculture with other cells. Differentiation into hematopoietic cells and dopaminergic neurons, for instance, were induced when mouse ES cells were replated on feeder layers of OP9 and PA6 cells, respectively ***(29,30)***.

4. Notes

1. It is acceptable to maintain ES cells without using feeder cells for a long term, if only in vitro work is planned without making knockout mice.
2. Currently, 10% of Knockout Serum Replacement (KSR, Gibco) and 1% FCS are used in the laboratory. ES cells can be maintained with better morphology in this condition.
3. PBS should be warmed to dissolve trypsin. Filter to sterilize, make aliquots, and store at –20ºC.
4. Alternatively, feeder cells can be treated with mitomycin C.
5. There are two alternative and easier methods available now for cloning genomic DNAs. BAC library DNA is commercially available (ResGen). The gene of interest can be screened using PCR, and the BAC plasmid containing the genomic DNA fragment can be purchased. Further, the genomic DNA can be directly PCR-amplified using sequence data available from mouse genome project (Celera).
6. Homozygous gene targeting can also be achieved by a consecutive targeting using a construct carrying another selection marker ***(31)***. Although less convenient, this consecutive method is considered to be more reliable to obtain double knockout ES cells (*see* **ref. *32***).

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References

1. Evans, M. J. and Kaufman, M. H. (1981) Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292,** 154–156.
2. Martin, G. R. (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. USA* **78,** 7634–7638.
3. Koller, B. H., Hageman, L. J., Doetschman, T. C., Hagaman, J. R., Huang, S., Williams, P. J., et al. (1989) Germline transmission of a planned alteration made in the hypoxanthine phosphoribosyltransferase gene by homologous recombination in embryonic stem cells. *Proc. Natl. Acad. Sci. USA* **86,** 8927–8931.
4. Thomas, K. R. and Capecchi, M. R. (1987) Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell* **51,** 503–512.
5. Kawasome, H., Papst, P., Webb, S., Keller, G. M., Johnson, G. L., Gelfand, E. W., and Terada, N. (1998) Targeted disruption of p70(s6k) defines its role in protein synthesis and rapamycin sensitivity. *Proc. Natl. Acad. Sci. USA* **95,** 5033–3038.
6. Doetschman, T. C., Eistetter, H., Katz, M., Schmidt, W., and Kemler, R. (1985) The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. *J. Embryol. Exp. Morphol.* **87,** 27–45.
7. Leahy, A., Xiong, J. W., Kuhnert, F., and Stuhlmann, H. (1999) Use of developmental marker genes to define temporal and spatial patterns of differentiation during embryoid body formation. *J. Exp. Zool.* **284,** 67–81.
8. Garrington, T. P., Ishizuka, T., Papst, P. J., Chayama, K., Webb, S., Yujiri, T., et al. (2000) MEKK2 gene disruption causes loss of cytokine production in response to IgE and c-Kit ligand stimulation of ES cell-derived mast cells. *EMBO J.* **19,** 5387–5395.
9. Cheng, A. M., Saxton, T. M., Sakai, R., Kulkarni, S., Mbamalu, G., Vogel, W., et al. (1998) Mammalian Grb2 regulates multiple steps in embryonic development and malignant transformation. *Cell* **95,** 793–803.
10. Rosen, E. D., Sarraf, P., Troy, A. E., Bradwin, G., Moore, K., Milstone, D. S., et al. (1999) PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro. *Mol. Cell* **4,** 611–617.
11. Smyth, N., Vatansever, H. S., Murray, P., Meyer, M., Frie, C., Paulsson, M., and Edgar, D. (1999) Absence of basement membranes after targeting the LAMC1 gene results in embryonic lethality due to failure of endoderm differentiation. *J. Cell Biol.* **144,** 151–160.
12. Kuang, W., Xu, H., Vachon, P. H., and Engvall, E. (1998) Disruption of the lama2 gene in embryonic stem cells: laminin alpha 2 is necessary for sustenance of mature muscle cells. *Exp. Cell Res.* **241,** 117–125.

13. Xu, C., Liguori, G., Adamson, E. D., and Persico, M. G. (1998) Specific arrest of cardiogenesis in cultured embryonic stem cells lacking Cripto-1. *Dev. Biol.* **196,** 237–247.
14. Vittet, D., Buchou, T., Schweitzer, A., Dejana, E., and Huber, P. (1997) Targeted null-mutation in the vascular endothelial-cadherin gene impairs the organization of vascular-like structures in embryoid bodies. *Proc. Natl. Acad. Sci. USA* **94,** 6273–6278.
15. Minamino, T., Yujiri, T., Papst, P. J., Chan, E. D., Johnson, G. L., and Terada, N. (1999) MEKK1 suppresses oxidative stress-induced apoptosis of embryonic stem cell-derived cardiac myocytes. *Proc. Natl. Acad. Sci. USA* **96,** 15,127–15,132.
16. Yujiri, T., Sather, S., Fanger, G. R., and Johnson, G. L. (1998) Role of MEKK1 in cell survival and activation of JNK and ERK pathways defined by targeted gene disruption. *Science* **282,** 1911–1914.
17. Hamazaki, T., Iiboshi, Y., Oka, M., Papst, P. J., Meacham, A. M., Zon, L. I., and Terada, N. (2001) Hepatic maturation in differentiating embryonic stem cells in vitro. *FEBS Lett.* **18,** 15–19.
18. Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S., and Jones, J. M. (1998) Embryonic stem cell lines derived from human blastocysts. *Science* **282,** 1145–1147.
19. Reubinoff, B. E., Pera, M. F., Fong, C. Y., Trounson, A., and Bongso, A. (2000) Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. *Nat. Biotechnol.* **18,** 399–404.
20. Wurst, W. and Joyner, A. L. (1992) Production of targeted embryonic stem cell clones, in *Gene Targeting, A Practical Approach* (Joyner, A. L., ed.), IRL, Toronto, Canada, pp. 33–61.
21. Hasty, P. and Bradley, A. (1992) Gene targeting vectors for mammalian cells, in *Gene Targeting, A Practical Approach* (Joyner, A. L., ed.), IRL, Toronto, Canada, pp. 1–31.
22. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Analysis of genomic DNA by southern hybridization, in *Molecular Cloning, A Laboratory Manual, Second edition*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 9.31–9.58.
23. Turksen, K. ed. (2002) Embryonic stem cells: methods an protocols, in *Methods Mol. Biol.* vol. 185, Humana Press, Totowa, NJ, pp. 1–499.
24. Yamashita, J., Itoh, H., Hirashima, M., Ogawa, M., Nishikawa, S., Yurugi, T., et al. (2000) Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors. *Nature* **408,** 92–96.
25. Klug, M. G., Soonpaa, M. H., Koh, G. Y., and Field, L. J. (1996) Genetically selected cardiomyocytes from differentiating embronic stem cells form stable intracardiac grafts. *J. Clin. Invest.* **98,** 216–224.
26. Soria, B., Roche, E., Berna, G., Leon-Quinto, T., Reig, J. A., and Martin, F. (2000) Insulin-secreting cells derived from embryonic stem cells normalize glycemia in streptozotocin-induced diabetic mice. *Diabetes* **49,** 157–162.
27. Muller, M., Fleischmann, B. K., Selbert, S., Ji, G. J., Endl, E., Middeler, G., et al. (2000) Selection of ventricular-like cardiomyocytes from ES cells in vitro. *FASEB J.* **14,** 2540–2548.

28. Andressen, C., Stocker, E., Klinz, F. J., Lenka, N., Hescheler, J., Fleischmann, B., et al. (2001) Nestin-specific green fluorescent protein expression in embryonic stem cell-derived neural precursor cells used for transplantation. *Stem Cells* **19,** 419–424.
29. Nakano, T., Kodama, H., and Honjo, T. (1996) In vitro development of primitive and definitive erythrocytes from different precursors. *Science* **272,** 722–724.
30. Kawasaki, H., Mizuseki, K., Nishikawa, S., Kaneko, S., Kuwana, Y., Nakanishi, S., et al. (2000) Induction of midbrain dopaminergic neurons from ES cells by stromal cell-drived inducing activity. *Neuron* **28,** 31–40.
31. te Riele, H., Maandag, E. R., Clarke, A., Hooper, M., and Berns, A. (1990) Consecutive inactivation of both alleles of the pim-1 proto-oncogene by homologous recombination in embryonic stem cells. *Nature* **348,** 649–651.
32. te Riele, H., Brouwers, C., and Dekker, M. (2000) Generation of double-knockout embryonic stem cells, in *Gene Knockout Protocols* (Tymms, M. J. and Kola, I., ed.), Humana Press, Totowa, NJ, pp. 251–262.

4

The Use of the Yeast Two-Hybrid System to Measure Protein–Protein Interactions that Occur Following Oxidative Stress

Richard A. Franklin

Abstract

Oxidative stress has been shown to have a myriad of effects on cells. Treatment of cells with oxidants, such as hydrogen peroxide or agents that induce reactive oxygen intermediates, has been shown to induce many cellular signaling pathways and, in some cases, cell apoptosis. Many chemotherapeutic treatments used to induce cell death do so via the induction of oxygen radicals. It is thought that oxidative stress can create, or modify the strength of, protein–protein interactions in cells that do not typically occur, or are weaker, under normal redox conditions. In this chapter, I describe a method to measure the strength of protein–protein interactions that may be enhanced during oxidative stress using the yeast two-hybrid system.

Key Words: Yeast two-hybrid; oxidative stress; cellular signaling; redox; protein-protein interactions.

1. Introduction

Treatment of cells with oxidants, such as hydrogen peroxide or agents that induce reactive oxygen intermediates, has been shown to induce several cellular signaling pathways. Certain cytokines, such as tumor necrosis factor-alpha (TNF-α), induce reactive oxygen intermediates and cell death ***(1)***. In addition, many chemotherapeutic treatments used to induce cell death in cancer do so via the induction of oxygen radicals ***(2–5)***. One report demonstrated that when Jurkat T lymphocytes were stimulated with TNF-α under normal culture conditions

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only minimal tyrosine phosphorylation was detectable. However, TNF-α treatment resulted in extensive tyrosine phosphorylation when the redox balance was shifted to a more oxidized intracellular environment using DL-buthionine-[S,R]-sulfoximine (BSO), an inhibitor of glutathione synthesis ***(6)***. These results demonstrate that the redox capacity of the cell can clearly influence the ability of a receptor to initiate signal transduction.

It was also demonstrated that oxygen radicals or ultraviolet (UV) light, which has been demonstrated to induce hydrogen peroxide generation in some cells ***(7)***, cause the aggregation of the TNF-receptor and activation of the JNK pathway ***(8)*** in the absence of any ligand. Thus, it appears that the oxidation-state of the cell can induce protein–protein interactions that normally do not occur. Because of this, we have developed a method using the yeast two-hybrid system that can be used to measure protein–protein interactions that occur during oxidative stress.

The two-hybrid system was developed by Fields and Song ***(9)*** and is an extremely sensitive method for identifying protein–protein interactions ***(10)***. Briefly, the two-hybrid system takes advantage of the properties of the GAL4 transcription factor. This transcription factor has separable domains for DNA binding (amino terminal) and transcriptional activation (carboxy terminal). A known gene can be inserted into a plasmid that will express a GAL4-binding-domain as a chimeric protein with any protein the investigator chooses to study (bait). A cDNA library, or known gene, can be cloned into another plasmid vector that will express a chimeric protein containing the GAL4-activation-domain with either a known or unknown protein (prey). The bait and prey vectors are transfected into yeast and colonies can be selected for the presence of both plasmids. If the chimeric bait protein interacts with the chimeric prey protein, transcription from the GAL4 promoter will occur (*see* **Fig. 1**). The GAL4 promoter, in this case, is coupled to the transcription of a reporter gene(s). The power of this system is that it is a sensitive means to identify protein–protein interactions that occur within an intact cell.

The intracellular redox capacity of yeast can be modulated by many of the same mechanisms as those used to modulate the redox capacity of mammalian cells (e.g., hydrogen peroxide and BSO) ***(11)***. This makes the yeast two-hybrid system applicable to study the influence of redox and oxygen radicals on protein–protein interactions.

2. Materials

2.1. Two-Hybrid System

1. Hybrizap 2.1 two-hybrid system (Stratagene, La Jolla, CA).
2. Plasmid CL1 (Clontech, Palo Alto, CA).

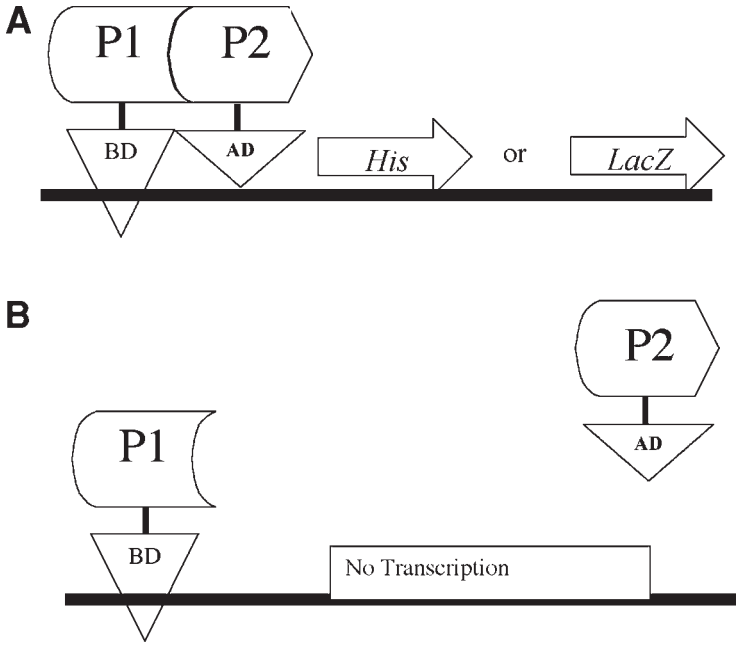

Fig. 1. A chimeric protein is expressed from the bait vector. This chimeric protein consists of one of your proteins of interest (P1) and a GAL4-binding domain (BD). A chimeric protein is also from the prey vector. This protein consists of your other protein of interest (P2) and a GAL4-activation domain (AD). (**A**) If protein P1 interacts with protein P2, then activation domain is localized to the GAL4 protomer and transcription occurs. (**B**) In the absence of an interaction of P1 and P2, the activation domain is not localized to the promoter and transcription does not occur. In the system used for these studies, the yeast have a *His* gene and a *LacZ* gene that have GAL4 promoters.

3. Assorted Restriction Enzymes (dependent on the proteins studied).
4. Qiagen plasmid prep columns (Qiagen, Valencia, CA).
5. Ampicillin (Sigma, St. Louis, MO).
6. Chloramphenicol (Sigma).
7. Gel boxes and power supply (for plasmid purifications).
8. Spectophotometer (for quantitation).
9. Water bath (for incubation).

2.2. Yeast Transfections and Selection

1. Salmon sperm (Sigma).
2. Dimethyl sulfoxide (DMSO) (Sigma).
3. 10X TE: 100 m*M* Tris-HCl, pH 7.5 (Sigma) and 10 m*M* ethylenediamine tetraacetic acid (EDTA) (Sigma).

4. 10X LiAc:1 *M* LiAc (Sigma) pH to 7.5 with acetic acid.
5. PEG 4000 Solution: 40% PEG 4000 (Sigma), 1X TE, and 1X LiAc.
6. 3-amino-1,2,4-triazole (3-AT; Sigma).
7. L-Leucine (Sigma).
8. L-Histidine hydrochloride (Sigma).
9. L-Tryptophan (Sigma).
10. Selective media (SM): 6.7 mg/mL of yeast nitrogen base (Difco, Detroit, MI), 2% glucose (Sigma), 20 µg/mL L-arginine HCl; 20 µg/mL adenine hemisulfate salt, 30 µg/mL L-isoleucine, 30 µg/mL L-lysine-HCl, 20 µg/mL L-methionine, 50 µg/mL L-phenylalanine, 200 µg/mL L-threonine, 30 µg/mL L-tyrosine, 150 µg/mL L-valine, and 20 µg/mL uracil; and for plates 15 gm/L Bacto Agar (Difco). All amino acids can be purchased from Sigma. When making this solution, the nitrogen base and agar are mixed together as a 2X stock and autoclaved. Once the mixture has cooled to 55ºC, sterile-filtered solutions of the amino acids and glucose can be added. We routinely made up 10X stock of amino acid supplement containing all the amino acids listed for the SM media. Glucose was also made up as a 10X stock (20% w/v). Water is added to create the 1X solution. Separate 10X stock solutions of histidine, threonine, and leucine are added in place of the appropriate amount of water as required. The glucose and amino acid stock solutions were kept at 4ºC. The 2X solution of yeast extract and agar was prepared right before use.
11. Environmental chamber with orbital shaking.
12. Spectophotometer.

2.3. Oxidative Stress of Yeast

1. Hydrogen peroxide (Sigma).
2. Optionally—DL-buthionine-[S,R]-sulfoximine (BSO; Sigma) (*see* **Note 1**).
3. 15 × 100 mm Snap top culture tubes (Fisher Scientific, Suwanee, GA).
4. Yeast-peptone dextrose media (YPD): 20 gm/L peptone (Difco) and 10 gm/L yeast extract (Difco). The mixture should initially be resuspended to 900 mL and autoclaved. The media should be left to cool to 55ºC and, at this point, 100 mL of sterile-filtered 20% glucose should be added. This solution should be kept at 4ºC.
5. Spectophotomer.
6. Environmental chamber with orbital shaking.

2.4. Yeast Cell Lysis

1. Glass beads (Sigma; 0.45–0.6 mm).
2. 250 m*M* Tris-HCl, pH 7.5 (Sigma).
3. Phosphate-buffered saline (PBS): Dissolve 256 mg of $NaH_2PO_4 \cdot H_2O$, 2.25 gm of $Na_2HPO_4 \cdot 7H_2O$, and 8.7 gm of NaCl into 900 mL of H_2O. (pH to 7.2. and adjust final volume to 1 L with H_2O).
4. Dry ice.
5. Ethanol.
6. Water bath.

2.5. β-Galactosidase Assay

1. o-Nitrophenyl-β-D-galactopyranoside (ONPG; Sigma).
2. ONPG Cleavage Buffer: 60 m*M* NaH_2PO_4, 40 m*M* Na_2HPO_4, 10 m*M* KCl, and 1 m*M* $MgSO_4$ (pH 7.0). All reagents for this buffer can be purchased from Sigma.
3. 1 *M* Na_2HCO_3 (Sigma).
4. Absorbance microtiter plate reader.

2.6. Protein Assay

1. BSA for preparation of standards (BSA; Sigma).
2. Bradford Reagent (Sigma). To prepare your own Bradford reagent, dissolve 100 mg Coomassie brilliant blue G-250 in 50 mL 95% ethanol, add 100 mL 85% (w/v) phosphoric acid. Dilute to 1 L when the dye has completely dissolved, and filter before use.
3. Absorbance microtiter plate reader.

3. Methods

3.1. Vectors

In our previously reported studies ***(12)***, we used the plasmid pGBT8 (*TRP1*$^+$), which encodes a GAL4-binding domain that is expressed from the ADH1 promoter when transformed into yeast and the plasmid pGAD-GL (*LEU2*$^+$), which encodes a GAL4-activation-domain that is expressed via a modified ADH1 promoter when transformed into yeast. These were purchased in a kit through Pharmingen (San Diego, CA). Although this particular kit is no longer commercially available, a similar yeast two-hybrid kit is available from Stratagene. The Pharmingen pGBT8 and pGAD vectors have been replaced by the pBD-GAL4-Cam (pBD-GAL4, *TRP*+) and pAD-GAL4-2.1 (pAD-GAL4, *LEU*+) vectors, respectively, in the Stratagene kit. The plasmid and bait vectors are slightly different in these two kits, however, the transcription of the chimeric proteins from both sets of vectors is driven by ADH1, they all encode chimeric proteins with either the GAL4 DNA-binding domain or GAL4-activation domain, and the amino acid deficiency, which both set of vectors complement, is identical. The one significant difference between these two sets of vectors is that the Stratagene pBD-GAL4 vector encodes chloramphenicol resistance, whereas, the pGBT8 vector encodes ampicillin resistance. Theoretically, any of the yeast two-hybrid systems currently available should work for these assays, however, proper controls in all two-hybrid systems need to be performed. The pCL1 (*LEU2*$^+$) vector encodes the intact GAL4 protein (Clontech, Palo Alto, CA) and represents an important control in these studies (*see* **Notes 2, 3**).

3.1.1. Vector Construction

The gene (or portion of the gene of interest) can be inserted into the multiple cloning site of either vector. In our previously published experiments, we inserted CDK4 into the bait vector and p16 into the prey vector ***(12)***. Great care must be taken in inserting the gene into the plasmid to ensure that it is oriented in the correct reading frame. If done correctly, transcription of the gene from the ADH1 promoter in yeast will yield a chimeric protein with either a GAL4 DNA binding domain (pGB-GAL4) or GAL4 transcriptional activating domain (pAD-GAL4) serving as the amino terminal. The presence of the insert should be verified by restriction digests. In addition, one can sequence across the junction of the GAL4 domain and your protein of interest to insure that it remains in the correct reading frame.

Once the vector is constructed, it is produced by transfecting it into DH5α *Escherichia coli*. These vectors contain a gene-encoding ampicillin (pAD-GAD4) or chloramphenicol (pBD-GAL4) resistance and the transfected bacteria can be selected based on this property. In our previously reported studies ***(12)***, we purified the different plasmid vectors from the bacteria using MAXI Prep kits available from Qaigen.

If one is unfamiliar with inserting cDNAs into plasmids and producing these plasmids in bacteria, they should refer to *Current Protocols in Molecular Biology* ***(13)*** or any of the other texts that cover this methodology.

3.2. Yeast Transfections and Selection

YRG-2 yeast, which is *TRP*$^-$ and *LEU*$^-$, can be used for these studies. This strain of yeast contains two reporter genes (*HIS3* and *lacZ*) each under the control of the GAL4 promoter. As a control, both the pBD-GAL4 and pAD-GAL4 vectors, containing their respective inserts, are transfected independently into yeast to ensure that the individual vectors (containing the inserted gene) are not sufficient by themselves to induce transcription of *HIS3*. Transfection of yeast is carried out as follows.

3.2.1. Single Transfections of Yeast

1. A 50-mL culture of YPD is inoculated with yeast cells.
2. This culture is incubated overnight at 30ºC with shaking at 225 rpm. The OD_{600} the next morning should be between 1–2.
3. Add an amount of the overnight culture to obtain an OD_{600} of 0.2 in a 300-mL volume of YPD. Incubate this culture an additional 3–4 h with shaking at 30ºC until the OD_{600} reaches 0.5.
4. Pellet the cells at 6000*g* for 5 min at room temperature and discard the supernatant.
5. Resuspend the pellet in 1.5-mL sterile 1X TE/LiAc made fresh from 10X TE and 10X LiAc.
6. Aliquot out 10 µg of plasmid DNA (bait or prey) into a microfuge tube on ice.

7. Add 20 µL of single-stranded salmon sperm (10 mg/mL stock) and mix carefully.
8. Add 200 µL of the resuspended yeast cells and 1.2 mL of PEG 4000 (40% PEG 4000, 1X TE, 1X LiAc), mix immediately without vortexing and incubate at 30ºC without shaking.
9. Add DMSO slowly to the tube to a final concentration of 10% (156 µL) and mix gently.
10. Incubate at 42ºC for 15 min. Chill on ice and then pellet the yeast cells at 6000*g* for 5 min at room temperature.
11. Aspirate off the supernatant and resuspend the cells in 1 mL of 1X TE. Plate out 100 µL of cells onto 10 cm or 300 µL of cells onto 15-cm plates.
12. Selection is carried out on plates prepared with SM media. For the pBD-GAL4 vector (with the inserted gene), this is done in the presence of 20 µg/mL of leucine and 10–25 m*M* 3-amino-1,2,4-triazole (3-AT). For the pAD-GAL4 vector (with the inserted gene), this is done in the presence of 20 µg/mL of tryptophan and 10–25 m*M* 3-AT. If tangible growth of the yeast is noted on these plates, it would suggest that the vectors are inducing transcription for the GAL4 promoter in the absence of protein–protein interactions and the system would have to be modified prior to continuing (*see* **Note 4**).
13. It is important that these transfections are controlled appropriately (*see* **Note 5**).

3.2.2. Cotransfection of Yeast

1. Once it is determined that your proteins of interest do not artifactully result in the transcription of the *HIS3* and *LacZ* genes, you can cotransfect both the pBD-GAL4 and pAD-GAL4 plasmids expressing your proteins of interest into the yeast. Cotransfection can be accomplished as outlined earlier, however, 10 µg of both the bait and prey plasmid are used in **step 6** of the single-transfection protocol (*see* **Subheading 3.2.1–6**).
2. Select the cotransfected yeast on plates similar to aforementioned, however, neither tryptophan, leucine, nor histidine are added to the SM plates (*see* **Notes 3 and 6**).
3. If no growth is noted on these plates, *see* **Notes 7 and 8**.

3.2.3. Transfection of pCL1

pCL1 transfections are carried out as outlined in **Subheading 3.2.1.** with the exception that pCL1 transfected yeast are selected on SM plates to which 20 µg/mL of L-tryptophan is added. For pCL1 transfected yeast cells, you would expect growth on histidine-lacking plates as the intact GAL4 promoter is present.

3.3. Oxidative Stress of Positive Colonies

The ability of oxidative stress to modulate protein–protein interactions can be performed in two ways. We term these methods the forward and reverse process. In the forward process, hydrogen peroxide (the oxidative stressor that we use, *see* **Note 9**) is added to one set of tubes and compared to a control in which no

hydrogen peroxide is added. In the reverse process, hydrogen peroxide is added to both the control and treatment cultures, the cultures are then washed, and placed back into media with or without or oxidative stressor.

3.3.1. For the Forward Process

1. Inoculum for the cultures is taken from selective plates of recently transfected cells. Cultures consisted of yeast transfected with plasmids expressing our proteins of interest and separate cultures of yeast transfected with pCL1.
2. Cultures for the forward treatment process are grown in selective media broth to an $OD_{600} \approx 1.0–2.0$. For pCL1 transfected cells, tryptophan is added to the media.
3. The cultures are diluted with SM broth to an $OD_{600} \approx 0.8$. Cultures are then aliquoted into tubes (15 × 100 mm), one set to remain untreated (negative control) and the other set is treated with 10 μ*M* hydrogen peroxide.
4. Following the treatments, the cells are subjected to a 1-h incubation at 30ºC, harvested by centrifugation at 1400*g* for 10 min and cellular lysates are prepared as outlined later.

3.3.2. For the Reverse Process

1. Cultures for the reverse treatment process are grown in selective media with the addition of 10 μ*M* hydrogen peroxide in the SM broth overnight. In this method, cells are being released from an oxidative stress to determine how this influences interactions between the bait and prey proteins. Similar to aforementioned for the pCL1 transfected cells, tryptophan is added to the broth.
2. The absorbance of the cultures is measured the next morning at 600 nm.
3. The cultures are spun at 1400*g* for 10 min and the pellets washed two times using SM broth.
4. After the final wash, the pellets are resuspended in the appropriate SM broth to a calculated $OD_{600} \approx 0.8$.
5. The cultures are then divided and the cells are subjected to a 1-h incubation at 30ºC, with or without the addition of hydrogen peroxide (10 μ*M*).
6. The cells are then harvested by centrifugation at 1400*g* for 10 min and cellular lysates are prepared as outlined later.

3.4. Preparation of Yeast Lysates

1. The pelleted yeast cells from the above treatment are resuspended in 3 mL of PBS and 1-mL aliquots are transferred to microcentrifuge tubes containing approx 25–35 glass beads/tube.
2. The samples are centrifuged at 14,000*g* for 10 min and the supernatant discarded.
3. Five freeze/thaw cycles are performed for each sample using dry ice/ethanol and a 37ºC water bath. Samples are vortexed vigorously for 5 s between each thaw and freeze.
4. The pellets are resuspended in 150 μL of 250 m*M* Tris-HCl (pH 7.4). Samples at this point are either assayed immediately or frozen at –20ºC for assay at a later time (*see* **Note 10**).

3.5. β-Galactosidase Assay

1. Samples are thawed, if previously frozen, and centrifuged at 14,000*g* for 10 min.
2. 20 µL of undiluted sample (from the supernatant avoiding the glass beads and any debris) are added to individual wells in a 96-well microtiter plate. As a negative control, 20 µL of 250 m*M* Tris-HCl or 20 µL of ONPG Cleavage Buffer are added to individual wells in a 96-well microtiter plate.
3. To the wells, containing either the samples or the controls, 80 µL of ONPG Cleavage Buffer are added.
4. Seventeen µL of ONPG solution (0.1 % w/v in water) is then added to the wells and the time of addition noted.
5. The plates are incubated (30ºC) until a noticeable yellow color develops in the wells containing lysates from the pCL1 cells (45–75 min).
6. To stop the reaction, 125 µL of 1 *M* Na_2HCO_3 are added to each well and the total time of incubation recorded.
7. The absorbance of the assay cultures is read at 410 nm.

3.6. Protein Determination

1. Total protein content of the sample is determined by performing dilutions of 1:2, 1:4, and 1:8 with distilled water.
2. 10 µL of the diluted samples is added to the well.
3. 10 µL of bovine serum albumin (BSA) standard at concentrations ranging from 1 mg/mL to 0.05 mg/mL is added to the plate to create a standard curve. Distilled water is added to two wells to serve as a negative control.
4. 200 µL of Bradford Reagent is then added to each well.
5. The plate is incubated at room temperature for 10 minutes and the absorbance read at 570 nm.
6. Protein content of the samples is determined using the linear regression of the standard curve.

3.7. Expression of Data

Miller units are used to define the strength of the bait prey interactions under the different redox states ***(14)***. The units of β-galactosidase activity are calculated as follows: [(OD_{410} × 380)/protein content/min]. The assumption that is made in these experiments is that the greater affinity the bait and prey have for each other, the increased transcription of the reporter gene.

4. Notes

1. Glutathione is one of the major antioxidants within the cell. Glutathione synthesis can be inhibited by incubating the cells with BSO. We have found that the addition of BSO into the cultures prior to experiments (1 m*M*) greatly enhanced interactions between p16 and cdk4.
2. Cotransfection of the empty bait vector and the prey vector containing the insert, as well as cotransfections of the empty prey vector with the insert containing bait

vector, are important controls to carry out. These control transfections should not grow in the absence of histidine.

3. The pCL1 control is needed because the redox capacity of the cell may modulate the binding of the transcription factor to the promoter of the gene, as well as induce novel or increased protein–protein interactions. Thus, it is important to compare your findings to the findings with the intact pCL1 protein with the finding observed using the proteins of interest.
4. If transfection of either the bait or the prey plasmid alone induces transcription from the reporter gene; a possible remedy for this would be to express a portion of the protein of interest in the chimeric molecule.
5. In **Subheading 3.2.1. step 12**, cells should also be placed on plates containing either leucine and histidine (pBD-GAL4) or tryptophan and histidine (pAD-GAL4) to ensure that the transfections were successful. Successful transfections should result in growth on these plates.
6. We found that recently transfected yeast worked best in our experiments.
7. If no growth is noted on plates containing yeast that have been cotransfected, one possibility is that the proteins of interest are not interacting. This can be tested by adding histidine to the plates and observing the plates for growth of yeast. Growth on these plates and not on histidine minus plates would suggest that your proteins do not interact.
8. If growth on plates is not obtained in the cotransfection experiments, another possibility is that the efficiency of transfection is too low. If this is the case, the yeast can be sequentially transfected. Singly transfected yeast cells can be obtained from the control plates discussed in **Subheading 3.8–2**. The yeast can then be prepared and transfected again as described in section **Subheading 3.2.1.** The yeast transfected with both vectors should grow in the absence of histidine, leucine, and tryptophan if the proteins of interest interact.
9. Other types of oxidative stress can be generated by the addition of nitric oxide generating compounds into the cultures.
10. To ensure that oxidative stress is not influencing the transcription or translation of the bait or prey plasmid, we performed Western blots. This would ensure that under the treatments, the expression of both the chimeric proteins remained similar under both control and experimental conditions.

References

1. Mayer, M. and Noble, M. (1994) N-acetyl-L-cysteine is a pluripotent protector against cell death and enhancer of trophic factor-mediated cell survival in vitro. *Proc. Natl. Acad. Sci. USA* **91,** 7496–7500.
2. Dvorakova, K., Payne, C. M., Tome, M. E., Briehl, M. M., McClure, T., and Dorr, R. T. (2000) Induction of oxidative stress and apoptosis in myeloma cells by the aziridine-containing agent imexon. *Biochem. Pharmacol.* **60,** 749–758.
3. Friesen, C., Fulda, S., and Debatin, K. M. (1999) Induction of CD95 ligand and apoptosis by doxorubicin is modulated by the redox state in chemosensitive- and drug-resistant tumor cells. *Cell Death Differ.* **6,** 471–480.

4. McLaughlin, K. A., Osborne, B. A., and Goldsby, R. A. (1996) The role of oxygen in thymocyte apoptosis. *Eur. J. Immunol.* **26,** 1170–1174.
5. Matroule, J. Y., Carthy, C. M., Granville, D. J., Jolois, O., Hunt, D. W., and Piette, J. (2001) Mechanism of colon cancer cell apoptosis mediated by pyropheophorbide-a methylester photosensitization. *Oncogene* **20,** 4070–4084.
6. Staal, F., Anderson, M., Staal, G., Herzenberg, L., Gitler, C., and Herzenberg, L. (1994) Redox regulation of signal transduction: tyrosine phosphorylation and calcium influx. *Proc. Natl. Acad. Sci. USA* **91,** 3619–3622.
7. Huang, C., Li, J., Ding, M., Leonard, S. S., Wang, L., Castranova, V., et al. (2001) UV induces phosphorylation of protein kinase B (Akt) at Ser473 and Thr308 in mouse epidermal Cl 41 cells through hydrogen peroxide. *J. Biol. Chem.* **276,** 40,234–40,240.
8. Rosette, C. and Karin, M. (1996) Ultraviolet light and osmotic stress: Activation of the JNK cascade through multiple growth factor and cytokine receptors. *Science* **274,** 1194–1197.
9. Fields, S. and Song, O. (1989) A novel genetic system to detect protein-protein interactions. *Nature* **340,** 245–246.
10. Finkel, T., Duc, J., Fearon, E., Dang, C., and Tomaselli, G. (1993) Detection and modulation in vivo of helix-loop-helix protein-protein interactions. *J. Biol. Chem.* **268,** 5–8.
11. Izawa, S., Inoue, Y., and Kimura, A. (1995) Oxidative stress response in yeast: effect of glutathione on adaptation to hydrogen peroxide stress in Saccharomyces cerevisiae. *FEBS Lett.* **368,** 73–76.
12. Martin, E. A., Robinson, P. J., and Franklin, R. A. (2000) Oxidative stress regulates the interaction of p16 with cdk4. *Biochem. Biophys. Res. Commun.* **275,** 764–767.
13. Chanda V. B. (1993) *Current Protocols in Molecular Biology,* Wiley, New York, NY, pp. 13.6.2–13.6.6.
14. Miller, J. (1972) *Experiments in Molecular Genetics,* Cold Spring Harbor Laboratory Press, Plainview, NY, pp. 352–353.

5

Differential Screening of cDNA Libraries for Analysis of Gene Expression During Tumor Progression

Christopher W. Gregory

Abstract

The ability to examine gene expression differences during cancer progression is crucial to our understanding of the biological events that lead to uncontrolled growth of malignant cells. Differential expression screening of complementary DNA libraries is described in detail here as a valuable method for monitoring gene expression changes in a prostate cancer xenograft model. Using Southern blot analysis of complementary DNA clones and Northern blot analysis to confirm differential expression, several androgen-regulated transcripts have been identified that may be important in prostate cancer progression.

Key Words: Gene expression; RNA; cDNA library; tumor progression; polymerase chain reaction (PCR); prostate cancer; xenograft; androgen; Southern blot analysis; Northern blot analysis.

1. Introduction

Changes in gene expression that lead to progression of disease have been studied using numerous methods, including reverse transcriptase polymerase chain reaction (RT-PCR), Northern blot analysis, differential display techniques, complementary DNA (cDNA) library screening, and, more recently, cDNA microarray hybridization. Detection of subtle and/or dramatic changes in mRNA expression levels is an important tool in studies focused on tumor progression.

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Steroid hormone-associated malignancies, such as breast and prostate cancer, are characterized by hormone-dependent and -independent phenotypes, with tumor progression following intermediate steps between the two states. Monitoring gene expression changes during this progression allows the identification of critical signaling pathways that may augment tumor growth. A novel method of differential expression screening of cDNA libraries in a human prostate cancer xenograft model is described here to illustrate the utility of such an approach as we attempt to identify androgen-regulated transcripts that may encourage prostate cancer tumor growth.

2. Materials

1. Tumor tissue or cells for RNA source.
2. Guanidine thiocyanate buffer: 4 *M* guanidine thiocyante, 16.7 m*M* sodium citrate, 0.5% sodium lauryl sarcosine, 0.1% antifoam A, 0.6% 2-mercaptoethanol (added fresh).
3. 5.7 *M* cesium chloride solution in 16.7 m*M* sodium acetate.
4. 3.0 *M* sodium acetate.
5. 70% and 100% ethyl alcohol.
6. RNA equilibration and wash buffer I: 10 m*M* Tris-HCl pH 7.5, 0.5 *M* NaCl, 1 m*M* ethylenediamine tetraacetic acid (EDTA), 0.5% sodium lauryl sarcosine.
7. RNA wash buffer II: 10 m*M* Tris-HCl pH 7.5, 0.5 *M* NaCl, 1 m*M* EDTA, 0.05% sodium lauryl sarcosine.
8. RNA elution buffer: 10 m*M* Tris-HCl pH 7.5, 1 m*M* EDTA, 0.05% sodium lauryl sarcosine.
9. Oligo-dT cellulose, Type 3.
10. Glyoxyl, 40% solution and dimethyl sulfoxide (DMSO).
11. Sodium phosphate buffer ($NaPO_4$), pH 6.8.
12. Biotrans nylon membrane (ICN, Costa Mesa, CA).
13. [^{32}P]dCTP (Amersham Corp., Arlington Heights, IL).
14. Prime-a-Gene System (Promega Corp., Madison, WI).
15. Standard sodium citrate (SSC) 20X: 3 *M* NaCl, 0.3 *M* Na_3citrate, pH 7.0.
16. Denhardt solution 100X: 2% Ficoll 400, 2% polyvinylpyrrolidone, 2% bovine serum albumin (BSA) in double-distilled H_2O (dd)H_2O.
17. Hybridization solution: 5X standard sodium citrate (SSC), 5X Denhardt solution, 1% sodium dodecyl sulfate (SDS), and 100 µg/mL salmon sperm DNA.
18. Wash solutions: 2X SSC-0.1% SDS and 0.1X SSC-0.1% SDS.
19. Tris/borate/EDTA (TBE) buffer, 10X: 890 m*M* Tris base, 890 m*M* boric acid, 20 m*M* EDTA, pH 8.0.
20. X-ray film for autoradiography.
21. Lambda phage ZAP II (Stratagene, La Jolla, CA).
22. Taq polymerase and buffer with $MgCl_2$ (Perkin-Elmer, Foster City, CA) and cloned plaque forming unit (PFU) enzyme (Stratagene).

23. Agarose gel electrophoresis equipment.
24. Polymerase chain reaction (PCR) instrument.

3. Methods

The methods outlined later describe: 1) preparation of mRNA from human prostate cancer tumor tissue; (2) construction of cDNA libraries; and 3) screening of libraries for differentially-expressed transcripts.

3.1. mRNA Isolation

1. Frozen CWR22 human prostate cancer xenograft tumors ***(1–3)*** from androgen-treated mice (androgen-dependent CWR22) and from mice castrated for 150 d (recurrent CWR22) were used to isolate total RNA as described ***(4)***. Briefly, tumor tissue was weighed and equal amounts (0.15–0.2 g) for each tumor were pulverized in liquid nitrogen using mortar and pestle.
2. Add 16 mL guanidine thiocyanate buffer per gram of tissue to the pulverized samples (*see* **Note 1**).
3. Homogenize immediately for 30 s with a Tissuemizer® or Polytron® device. Wash homogenizer with sterile water between samples and wet with guanidine before each new sample.
4. Centrifuge tubes at 500*g* at 10ºC for 15 min.
5. Add 8 mL of 5.7 *M* CsCl per gram of tissue homogenized to ultracentrifuge tubes (SW41 or smaller capacity SW50.1). Layer supernatant from homogenate carefully onto CsCl cushion using a sterile pipet. Fill tubes to within 4 mm of top. Balance tubes and centrifuge at 150,000*g* for 16–20 h at 25ºC.
6. In a chemical fume hood, remove guanidine with sterile pipet down to level of CsCl. Remove CsCl by pipet, avoiding translucent pellet in bottom of tube. Invert the tube to drain excess CsCl solution. Cut off top of each tube with clean razor blade leaving about 2 cm at bottom.
7. Dissolve the pellet in 200–400 µL sterile deionized distilled water and transfer to a sterile 1.5-mL microfuge tube.
8. Add 40 µL 3 *M* sodium acetate (pH 5.5) and 1 mL absolute ethanol.
9. Allow precipitate to form overnight at –20ºC.
10. Centrifuge 15 min in microfuge and decant supernatant. Wash pellet with 70% ethanol by adding 1 mL and briefly vortexing to release pellet from tube.
11. Centrifuge at 5000 rpm for 5 min and decant ethanol. Invert tube and allow to air-dry for 15–30 min.
12. Redissolve pellet in 200–400 µL sterile deionized distilled water and mix by trituration to resuspend RNA. Remove 5 µL, add to 495 µL water and determine concentration of RNA by determining absorbance at 260 and 280 nm in a spectrophotometer.

3.2. cDNA Library Construction

The construction of cDNA libraries is described in **Subheadings 3.2.1.–3.2.5.** This includes: a) poly-A+ mRNA isolation; b) cDNA synthesis; c) ligation and

kinasing of adapters; d) size fractionation of cDNAs; and e) ligation of cDNAs into lambda phage and amplification of libraries.

3.2.1. Isolation of Poly-A+ mRNA from Total Tumor RNA

PolyA+ mRNA was isolated as described ***(5)***. Pour a 1–2-mL column of oligo-dT cellulose (Type-3) in 0.1 *M* NaOH and allow cellulose to settle. Wash the column with five column volumes of 0.1 *M* NaOH and five bed volumes of sterile ddH_2O. Equilibrate the column with 10 bed volumes of RNA equilibration buffer. Heat 1–2 mg of total RNA to 65ºC for 5 min and allow to cool to room temperature. Add 5 *M* NaCl and 10% sodium lauryl sarcosine to final concentrations of 0.5 *M* and 0.5%, respectively. Apply the sample to the column, collect the flowthrough, and warm it again to 65ºC, cool, and reapply to column. Wash the column with five bed volumes of wash buffer I and then wash with five column volumes of wash buffer II. To elute the RNA, use 400 µL of elution buffer twice and save in separate tubes on ice. Add 0.1 volume 3 *M* sodium acetate and two volumes of absolute ethanol to precipitate the RNA overnight at –20ºC. Collect the RNA by centrifugation in a microfuge at top speed for 30 min. Dissolve the RNA in an appropriate volume of sterile ddH_2O.

3.2.2. cDNA Synthesis

Following the manufacturer's instructions, use 5 µg poly A+ RNA for first strand cDNA synthesis. After 1 h incubation at 37ºC, remove samples to ice and prepare samples for second strand synthesis. Add [^{32}P]αdCTP, RNase H and DNA polymerase I and incubate for 2.5 h at 16ºC. Return the samples to ice and add blunting deoxynucleotide 5'-triphosphate (dNTP) mix and cloned PFU enzyme. Incubate at 72ºC for exactly 30 min. Add 200 µL phenol:chloroform (1:1) and vortex for 1 min followed by 2 min centrifugation at room temperature. Remove aqueous phase and repeat phenol extraction twice more (*see* **Note 2**). Precipitate cDNA in aqueous solution by adding 0.1 vol of 3 *M* sodium acetate and two volumes of absolute ethanol. Store overnight at –20ºC.

3.2.3. Ligation and Kinasing of Adaptors

Centrifuge for 1 h at 4ºC to pellet DNA. Remove the radioactive supernatant and save temporarily. Wash the pellet with 500 µL 70% ethanol, centrifuge briefly, decant ethanol, and air-dry the pellet. Resuspend cDNA pellets in *Eco*RI adaptor solution and incubate 30 min on ice after mixing by pipet. Remove a 1-µL aliquot and save at –20ºC as second strand cDNA for agarose gel electrophoresis. Add T4 DNA ligase enzyme, buffer and nucleotides and incubate at 4ºC for 48 h. Heat the ligation reaction at 70ºC for 30 min to destroy ligase enzyme. Spin briefly, cool to room temperature, and add kinase buffer, nucleotides, and T4 DNA kinase. Incubate samples at 37ºC for 30 min followed by 70ºC for 30 min

to destroy kinase. Spin briefly and cool to room temperature. Perform restriction enzyme digestion by adding *Xho*I buffer and *Xho*I enzyme and incubating 1.5 h at 37ºC. Cool to room temperature and add 5 µL TE buffer and store at –20ºC overnight.

3.2.4. Size Fractionation of DNA

Prepare a DNA sizing column (SizeSep 400, Pharmacia) according to manufacturer's directions. Load DNA sample onto center of column bed and centrifuge at 400*g* for 2 min and collect the flowthrough in a 1.5-mL tube. Save 5 µL of each sample after each wash and centrifugation. Wash the column twice with 60 µL of STE (10 m*M* Tris-HCl, pH 7.5, 10 m*M* NaCl, 1m*M* EDTA, pH 8.0) and perform one phenol:chloroform and one chloroform extraction on the samples. Precipitate the DNA by adding 0.1 volume of 3 *M* sodium acetate and two volumes of absolute ethanol. Store at –20ºC overnight.

To determine relative sizes of DNAs, pour a 6% TBE acylamide large format gel and electrophorese the 5-µL samples saved earlier for 2 h at 100 V. Dry the gel and expose to X-ray film overnight.

Centrifuge the cDNA fractions saved from the columns for 1 h at room temperature and wash the pellets with 100 µL 70% ethanol. Decant the ethanol and air-dry the pellets completely. Resuspend each pellet in 10 µL ddH_2O. Spot control DNA (0–200 ng) onto ethidium bromide containing agarose plate and spot 1 µL of each cDNA onto the plate. Photograph the plate upside down under ultraviolet (UV) light. Use the photograph to estimate the quantity of DNA represented in each sample by comparing to the titrated standard DNA.

3.2.5. Ligation of cDNA Inserts into Lambda Phage and Library Amplification

Using 100–300 ng of cDNA inserts, ligate to lambda phage arms (λ uni-ZAP XR, Stratagene) for 48 h at 4ºC. Store ligation samples at –20ºC (*see* **Note 3**).

To package the cDNA inserts, thaw four tubes of Gigapack III until just thawed and add 1 µL of ligation to each tube, mix, and centrifuge briefly. Incubate the samples at room temperature for exactly 2 h and then add 500 µL of SM ***(6)*** and 20 µL of chloroform. Invert to mix and centrifuge for 2 min.

Prepare NZY bacterial growth plates (150 mm) and top agar as described ***(6)***. Store top agar at 48ºC before use. Inoculate and grow a 100-mL culture of XL1BlueMRF' bacteria. Monitor cell growth by optical density measurements at 600 nm (A_{600}). Cells should be used when A_{600} reaches 0.5. Mix 100 µL of phage with 600 µL of bacteria and incubate at 37ºC for 15 min with gentle shaking. Add 6.5 mL of top agar (48ºC) and pour onto 150-mm NZY plate at 37ºC. Cool at room temperature until solid, place plates into 37ºC incubator, and reduce temperature to 33ºC. Incubate overnight.

To elute the phage from the plates, add 10 mL of cold SM to each plate, swirl, and collect. Rinse each plate with 1 mL SM. Pool the eluants into a sterile tube and store on ice. Determine the volume and add chloroform to 5% and mix. Incubate 15 min at room temperature and centrifuge for 10 min at 2500*g*. Mix 115 mL of stock with 9 mL of DMSO and freeze in 1.5-mL aliquots at –80ºC. Add chloroform to remaining sample to final concentration of 0.3% and store at 4ºC.

To titer the final amplified library, assume that there are approx 10^7 PFU/µL and perform 1:10 serial dilutions beginning with 10^3 (1 µL in 1 mL) to 10^9. Plate 10 µL per 100-mm plate. Incubate at 37ºC overnight and count the number of colonies the following day on each plate.

3.3. cDNA Library Screening

Screening of the cDNA libraries will be described under **Subheadings 3.3.1.–3.3.3.** and consists of amplification of clones using PCR, Southern blot analysis, and confirmation of differential expression by Northern blot analysis.

3.3.1. Amplification of Clones

Dilute 1 µL of stock cDNA library in 1.5-mL SM and plate onto a 10-cm LB plate. Repeat for up to 10 plates. Incubate plates at 37ºC overnight. Prepare 1.5-mL Eppendorf tubes by adding 500 µL SM and 20 µL chloroform and label appropriately including library name and plaque numbers. Using a yellow tip, pick two individual plaques into each tube and store tubes at 4ºC until amplification (*see* **Note 4**).

Prepare for PCR by labeling PCR tubes with phage numbers. Do 28 samples per run (or the maximum that PCR instrument can accomodate) with one blank tube. Add 10 µL of each phage stock to PCR tubes first and keep tubes on ice. Prepare PCR mixture (for one sample add: 6 µL 10X PETAQ with Mg^{++}, 0.6 µL each of T3 and T7 primers at 50 pmol/µL, 0.6 µL 10 m*M* dNTPs, 41.8 µL ddH_2O, 0.3 µL *TAQ* enzyme at 5 U/µL, and 0.1 µL cloned PFU at 2.5 µL). Add 50 µL PCR mix to each tube on ice and cover with 50 µL light mineral oil.

Perform PCR with the following conditions: heat the block to 95ºC in order to have a "hot start" and PCR at 95ºC for 3 min, 50ºC for 3 min, and 68ºC for 15 min for one cycle followed by 95ºC for 30 s, 50ºC for 1 min, and 72ºC for 3 min for 36 cycles followed by 72ºC for 5 min. After PCR, add 50 µL chloroform and 5 µL 10X TBE gel-loading buffer with bromophenol blue dye. Vortex, centrifuge briefly, and store at –20ºC until Southern blotting is performed.

3.3.2. Southern Blot Analysis

Prepare a 100-mL 1.5% agarose 1X TBE gel and pour into a 16 cm × 11 cm (or comparable) gel box and use two 30-tooth combs to cast an upper and lower

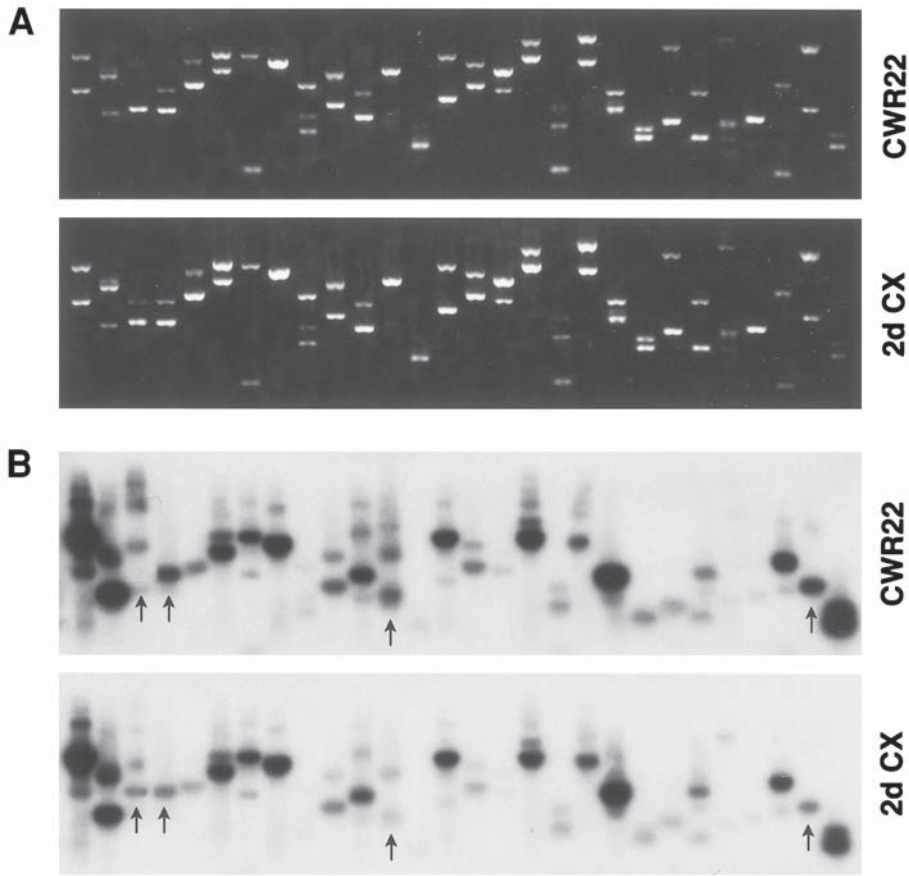

Fig. 1. Representative agarose gels and Southern blots from CWR22 cDNA library screening. cDNA clones were amplified by PCR and 12-µL samples were subjected to electrophoresis in 1.5% agarose 1X TBE gels. Gels were photographed under UV light (**A**) and then DNAs transferred to nylon membranes. [^{32}P]-labeled first-strand cDNAs synthesized from androgen dependent CWR22 tumor RNA (CWR22) or CWR22 tumor from mice castrated for 2 d (2d CX) were used for hybridization with membranes. Membranes were washed and exposed to X-ray film (**B**). Differentially expressed DNAs are designated by arrows.

gel. Add 2.5 µL 10 mg/mL ethidium bromide to gel just before pouring. Load 12 µL samples per lane in duplicate (top gel identical to bottom gel) and load a molecular weight marker into the remaining well. Electrophorese the DNA at 100 V for 1.5 h in 1X TBE buffer with ethidium bromide. Upon completion, photograph the gel on a UV light box (*see* **Fig. 1A**) and then incubate the gel in 250 mL 0.4 *M* NaOH for 20 min with gentle shaking. Assemble a standard

upward capillary transfer ***(6)*** in 0.4 *M* NaOH, using positively charged nylon membrane prewetted in ddH_2O. Use 300 mL ddH_2O in a 500-mL bottle as a weight on the transfer. After overnight transfer, rinse the membrane in ddH_2O for 1 min and in 2X standard sodium citrate for 5 min. Soak the membrane in 5% acetic acid for 2 min prior to staining with aqueous solution of methylene blue. Destain with multiple ddH_2O rinses. Mark the wells with a pencil and label the top and bottom of the membranes and mark the molecular weights from the marker. Cut the membrane into top and bottom halves.

Messenger RNA (polyA+) is isolated as described in **Subheading 3.2.1.** from tumors of interest (in this case, androgen-dependent CWR22 tumor and tumor from mice castrated 2 d earlier). First-strand cDNA labeled with [^{32}P]dCTP is produced using reverse transcription. Mix 1 µg of mRNA with 10 µL enzyme buffer, 2 µL random oligonucleotide primers (1.5 µg/µL), 5 µL dNTPs (2 m*M* dAGT and 0.2 m*M* dCTP), 5 µL 100 m*M* dithiothreitol (DTT), and 22 µL ddH_2O. Anneal the primers by incubating 10 min at room temperature and then add 5 µL [^{32}P]dCTP and 1 µL MMLV reverse transcriptase enzyme (200 U). Incubate for 1 h at 37ºC. Add 50 µL 0.6 *M* NaOH-50 m*M* EDTA and incubate for 30 min at 65ºC. Add 40 µL 1 *M* HEPES (free acid) and remove unincorporated nucleotides using a BioRad BioSpin column. Determine counts per min/µL sample using scintillation counter or handheld Geiger monitor.

While labeling RNA samples, prehybridize the Southern blot membranes of interest for 2–4 h at 68ºC in the following buffer: 5X SSC, 5X Denhardt solution, 1% SDS, 100 µg/mL salmon sperm DNA. Prehybridize 2–4 membranes per roller bottle or bag. Organize blots such that the "top" blots of each set are together and the "bottom" blots (duplicates) are grouped together.

Add labeled first-strand cDNAs to fresh hybridization buffer at 68ºC (approx 10 cpm/µL). Hybridize the "top" membranes with one cDNA and the "bottom" membranes with the other cDNA. Incubate at 68ºC overnight. After hybridization, decant the labeling buffer into radioactive waste container and briefly rinse the membranes in 2X SSC–0.1% SDS at room temperature followed by 0.1X SSC–0.1% SDS at 50ºC for 1 h. Expose the membranes to X-ray film. Compare duplicate blot sets probed with different cDNAs to determine those clones that are differentially expressed (e.g., strong band for specific clone when probed with cDNA from one tumor and weak or no band for same clone when probed with cDNA from another tumor) (*see* **Fig. 1B**). Clones that are differentially expressed may be selected for further Southern analysis to confirm expression (*see* **Notes 5, 6**).

3.3.3. Confirmation of Differential Expression by Northern Analysis

To confirm the differential expression of a specific DNA of interest, Northern blotting of RNAs from different tumors is used. Prepare total RNA as described

in **Subheading 3.1.** from tumors of interest (those used for cDNA synthesis originally, as well as other tumors that may provide evidence of expression differences).

Prepare 1% agarose gels in 10 m*M* $NaPO_4$ buffer, pH 6.8. Aliquot 10 µg samples of RNAs of interest and lyophilize under vacuum. Prepare glyoxylation mix with freshly deionized DMSO (100% solution) and glyoxal (40% solution). For 50 RNA samples, mix 250 µL of DMSO, 100 µL of glyoxal, and 150 µL of 0.067 *M* $NaPO_4$, pH 6.8. Add 10 µL of glyoxylation mix per RNA sample, mix by fingertip, centrifuge briefly, and incubate at 50ºC for 1 h. Prepare loading dye with 100 µL 50% sucrose, 20 µL xylene cyanol saturated solution and 20 µL bromophenol blue saturated solution. After 1 h incubation of RNA samples, centrifuge briefly and transfer to ice. Add 2.5 µL dye per sample. Load samples onto gel and electrophorese at 4 V/cm constant voltage in 10 m*M* $NaPO_4$, pH 6.8 buffer. Run the bromophenol blue dye at least half way on gel. Set up a standard upward capillary transfer (as described in **Subheading 3.3.2.**) using 20X SSC as transfer buffer and prewetted nylon membranes. Following overnight transfer, air-dry the membranes and UV crosslink for 1 min. Membranes may be stained with methylene blue as described in **Subheading 3.3.2.** to visualize lanes and 18 and 28S rRNA bands. Mark membranes appropriately with date, name, and markers at rRNA bands.

To generate labeled DNAs for hybridization of Northern membranes, perform PCR amplification as described in **Subheading 3.3.1.** using 10 µL of each DNA of interest. After PCR, add 100 µL chloroform, vortex to mix, centrifuge briefly, and remove the aqueous phase to a fresh tube. Precipitate the DNA by adding 2.4 µL 5 *M* NaCl and 120 µL absolute ethanol and storing overnight at −20ºC. Centrifuge to pellet DNA, resuspend in 25 µL ddH_2O, and electrophorese 1 µL DNA on 1.2% agarose-*tris* acetate-EDTA gel. Photograph gel on UV light box and estimate quantity of DNA by comparing to standard DNA in molecular weight ladder. Use 10 ng of DNA for random prime labeling with [^{32}P]dCTP. 10 ng DNA is combined with ddH_2O up to 5 µL and heated at 95ºC for 5 min and placed on ice. Reaction mix, dNTPs, [^{32}P]dCTP and Klenow enzyme are added and incubated at room temperature for 1 h. Add 1 *M Tris*-EDTA, pH 8.0 to bring volume to 100 µL and separate unincorporated nucleotides using a spin column as described in **Subheading 3.3.2.** Determine counts per minute/µL sample by scintillation counting or using a handheld Geiger monitor. Hybridize and wash membranes as described in **Subheading 3.3.2.** Expose membranes to X-ray film and compare expression of genes in different tumors (*see* **Notes 7, 8**).

As differentially expressed genes are identified, the DNAs are sequenced using M13 forward and reverse primers. DNA sequences are then compared to sequences in GenBank (National Center for Biotechnology Information, Bethesda, MD) to determine if the gene of interest is novel or previously described. Of 1652 cDNAs

screened from CWR22 xenografts, we identified 24 transcripts as being differentially expressed as tumors progressed from androgen dependent to androgen independent stages (***3***).

4. Notes

1. Smaller tissue pieces (>0.15 g) can be homogenized in guanidine thiocyanate without pulverization.
2. After cDNA synthesis and labeling, take appropriate precautions in handling all supernatants that contain [^{32}P]-labeled nucleotides. Save the supernatants until certain that cDNAs are recovered.
3. A test packaging and plating of ligation products is recommended to determine percent recombinant and titer of the library.
4. cDNAs may be further purified by diluting phage stocks 1:10 up to 1:1000 in SM and replating. Pick multiple single colonies from each plate and repeat PCR amplification. Compare PCR products to those from pooled phage stocks.
5. Southern blots may be stripped of [^{32}P]-labeled cDNAs for reprobing by treating membranes with 30 m*M* NaOH solution for 10 min with shaking followed by three 10-min TE washes.
6. A secondary Southern blot screening may be performed to confirm differential expression before proceeding to Northern blot analysis.
7. Northern blot membranes can be stripped of [^{32}P]-labeled cDNAs for reprobing by treating membranes for 20 min in boiling 0.1% SDS. Heat SDS solution to boiling, pour onto membrane, and shake for 20 min, allowing solution to cool. Expose membranes to X-ray film to determine if [^{32}P] signal is decreased.
8. For quantitative analysis of differences in expression, Northern blot membranes should be rehybridized with a housekeeping gene cDNA (e.g., 18S rRNA or actin). Only perform this re-hybridization after blots have been used for all clones of interest.

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References

1. Wainstein, M. A., He, F., Robinson, D., Kung, H.-J., Schwartz, S., Giaconia, J. M., et al. (1994) CWR22: androgen-dependent xenograft model derived from a primary human prostatic carcinoma. *Cancer Res.* **54,** 6049–6052.

2. Nagabhushan, M., Miller, C. M., Pretlow, T. P., Giaconia, J. M., Edgehouse, N. L., Schwartz, S., et al. (1996) CWR22: the first human prostate cancer xenograft with strongly androgen-dependent and relapsed strains both in vivo and in soft agar. *Cancer Res.* **56,** 3042–3046.
3. Gregory, C. W., Hamil, K. G., Kim, D., Hall, S. H., Pretlow, T. G., Mohler, J. L., and French, F. S. (1998) Androgen receptor expression in androgen-independent prostate cancer is associated with increased expression of androgen-regulated genes. *Cancer Res.* **58,** 5718–5724.
4. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979) Isolation of biologically active RNA from sources enriched in ribonucleases. *Biochemistry* **18,** 5294–5299.
5. Hamil, K. G. and Hall, S. H. (1994) Cloning of rat Sertoli cell follicle-stimulating hormone primary response complementary deoxyribonucleic acid: regulation of TSC-22 gene expression. *Endocrinology* **134,** 1205–1212.
6. Greenstein, D., Besmond, C., Ellington, H., and Pollard, J. P. (1998*) Current Protocols in Molecular Biology*, Vol. 1 (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds.). Wiley, New York, pp. 1.0.1–2.12.7.

6

Mitogen-Activated Protein Kinase Signaling in Drug-Resistant Neuroblastoma Cells

Raymond R. Mattingly

Abstract

Widespread inherent or acquired resistance to cytotoxic drugs is a major limitation to chemotherapy. There are many mechanisms that contribute to such resistance. In neuroblastomas there is evidence that acquired drug resistance may be associated with altered response to growth factor signals. The ubiquitous mitogen-activated protein kinase (MAPk) cascade, which transmits growth factor signals from the cell membrane to the nucleus, provides a principal mechanism for regulation of cell cycle progression and proliferation. We have shown that there is a relationship between acquired drug resistance in human neuroblastoma cells to doxorubicin, a topoisomerase-2 inhibitor, and to MDL-28842, an inhibitor of S-adenosylhomocysteine hydrolase, and reductions in the activation and nuclear translocation of MAPk.

Key Words: Neuroblastoma; MAP kinase; ERK; epidermal growth factor; confocal immunofluorescence; nuclear localization; doxorubicin.

1. Introduction

The ubiquitous mitogen-activated protein kinase (MAPk) cascade, which transmits growth factor signals from the cell membrane to the nucleus ***(1)***, provides a principal mechanism for regulation of cell-cycle progression and proliferation ***(2)***. Constitutive and inappropriate activation of MAPk may be a critical component in some human tumors ***(3,4)***, and thus pharmacological intervention in the MAPk cascade has been identified as a promising new approach to cancer

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therapy ***(5)***. Widespread inherent or acquired resistance to cytotoxic drugs is a major limitation to chemotherapy ***(6)***. We have shown that there is a relationship between acquired drug resistance in human neuroblastoma cells to doxorubicin, a topoisomerase-2 inhibitor ***(7)***, and to MDL-28842, an inhibitor of *S*-adenosylhomocysteine hydrolase ***(8)***, and the activation and nuclear translocation of MAPk ***(9)***.

The activation of MAPk is dependent on its dual phosphorylation at both Threonine-183 and Tyrosine-185 residues ***(10)***. Commercial availability of antibodies that recognize this dually phosphorylated and active form of MAPk with very great selectivity over the dephosphorylated or monophosphorylated forms provides straightforward assay protocols for the activation of MAPk by both Western blotting and immunofluorescence. Confocal immunofluorescence detection allows confirmation that the activated MAPk has been translocated to the nucleus.

2. Materials

2.1. Cell Culture and Lysis

1. Dulbecco's Modified Eagle's Medium (DMEM) (Gibco/BRL, Bethesda, MD) supplemented with 10% fetal bovine serum (FBS, HyClone, Ogden, UT).
2. Doxorubicin (Sigma, St. Louis, MO) is dissolved in tissue-culture water at 10 m*M*, stored in aliquots at −80ºC, and then added to tissue culture dishes as required.
3. Solution of trypsin (0.25%) and ethylenediamine tetraacetic acid (EDTA) (1 m*M*) from Gibco/BRL.
4. Epidermal growth factor (EGF, Gibco) is dissolved at 1 mg/mL in DMEM and stored in single use aliquots at −80ºC. Working solutions are prepared by dilution in 100 µg/mL BSA.
5. Phorbol 12-myristate 13-acetate (PMA, Sigma) is dissolved at 2 m*M* in dimethyl sulfoxide (DMSO) and stored in single use aliquots at −80ºC.
6. Modified Laemmli ***(11)*** buffer for cell lysis: 75 m*M* Tris-HCl, pH 6.8, 1.5% (w/v) sodium dodecyl sulfate (SDS), 7.5 % (w/v) glycerol, 200 m*M* β-mercaptoethanol, 0.03% (w/v) bromophenol blue, 0.003% (w/v) pyronin-Y. Store in aliquots at −20ºC. (*See* **Note 1**).
7. Teflon cell scrapers (Fisher).

2.2. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Separating buffer (4X): 1.5 *M* Tris-HCl, pH 8.7, 0.4% SDS. Store at room temperature.
2. Stacking buffer (4X): 0.5 *M* Tris-HCl, pH 6.8, 0.4% SDS. Store at room temperature.
3. Thirty percent acrylamide/bis solution (37.5:1 with 2.6% C) (this is a neurotoxin when unpolymerized and so care should be taken not to receive exposure) and N,N,N,N'-Tetramethyl-ethylenediamine (TEMED, Bio-Rad, Hercules, CA) (*see* **Note 2**).

4. Ammonium persulfate: prepare 10% solution in water and immediately freeze in single use (200 µL) aliquots at –20ºC.
5. Water-saturated isobutanol. Shake equal volumes of water and isobutanol in a glass bottle and allow to separate. Use the top layer. Store at room temperature.
6. Running buffer (5X): 125 m*M* Tris, 960 m*M* glycine, 0.5% (w/v) SDS. Store at room temperature.
7. Prestained molecular weight markers: Kaleidoscope markers (Bio-Rad, Hercules, CA).

2.3. Western Blotting for Active MAPk

1. Setup buffer: 25 m*M* Tris (do not adjust pH), 190 m*M* glycine, 20% (v/v) methanol.
2. Transfer buffer: Setup buffer plus 0.05% (w/v) SDS. Store in the transfer apparatus at room temperature (with cooling during use, *see* **Note 3**).
3. Supported nitrocellulose membrane from Millipore, Bedford, MA, and 3MM Chr chromatography paper from Whatman, Maidstone, UK.
4. Tris-buffered saline with Tween (TBS-T): Prepare 10X stock with 1.37 *M* NaCl, 27 m*M* KCl, 250 m*M* Tris-HCl, pH 7.4, 1% Tween-20. Dilute 100 mL with 900-mL water for use.
5. Blocking buffer: 5% (w/v) nonfat dry milk in TBS-T.
6. Primary antibody dilution buffer: TBS-T supplemented with 2% (w/v) fraction V bovine serum albumen (BSA).
7. Antidually phosphorylated MAPk ***(12)*** (available from Sigma, *see* **Note 4**).
8. Secondary antibody: Antimouse IgG conjugated to horse radish peroxidase (Santa Cruz, Santa Cruz, CA).
9. Enhanced chemiluminescent (ECL) reagents from Kirkegaard and Perry (Gaithersburg, MD) and Bio-Max ML film (Kodak, Rochester, NY) (*see* **Note 5**).

2.4. Stripping and Reprobing Blots for Total MAPk

1. Stripping buffer: 62.5 m*M* Tris-HCl, pH 6.8, 2% (w/v) SDS. Store at room temperature. Warm to working temperature of 70ºC and add 100 m*M* β-mercaptoethanol.
2. Wash buffer: 0.1% (w/v) BSA in TBS-T.
3. Primary antibody: Anti-ERK (Transduction, Lexington, KY).

2.5. Confocal Immunofluorescence for Active and Total MAPk

1. Microscope cover slips (22 × 40 × 0.15 mm) from Fisher, Pittsburgh, PA, and Lab-Tek two-well glass chamber slides from Nalge Nunc, Naperville, IL.
2. Phosphate buffered saline (PBS): Prepare 10X stock with 1.37 *M* NaCl, 27 m*M* KCl, 100 m*M* Na_2HPO_4, 18 m*M* KH_2PO_4 (adjust to pH 7.4 with HCl if necessary) and autoclave before storage at room temperature. Prepare working solution by dilution of one part with nine parts water.
3. Paraformaldehyde (Fisher): Prepare a 4% (w/v) solution in PBS fresh for each experiment. The solution may need to be carefully heated (use a stirring hot-plate in a fume hood) to dissolve, and then cool to room temperature for use.

4. Quench solution: 50 m*M* NH_4Cl in PBS.
5. Permeabilization solution: 0.5% (v/v) Triton X-100 in PBS.
6. Antibody dilution buffer: 3% (w/v) BSA in PBS.
7. Secondary antibody: Antimouse IgG conjugated to Cy3 (Jackson, West Grove, PA).
8. Nuclear stain: 300 n*M* DAPI (4,6-diamidino-2-phenylindole) in water.
9. Mounting medium: Antifade (Molecular Probes, Eugene, OR).

3. Methods

The active, dually phosphorylated form of MAPk is inherently labile owing to protein phosphatase activities within the cell. To obtain reliable and reproducible results, therefore, it is important to terminate the samples rapidly and effectively at the end of the treatment protocol. The antibody to the active, dually phosphorylated form of MAPk is sufficiently sensitive to allow detection in relatively small amounts of whole cell lysates, allowing the samples to be prepared by addition of boiling cell lysis buffer. Confirmation of equal recovery of the samples through the procedure is provided at the end by reprobing the blots for the total amount of MAPk that is present (phosphorylated or not). Treatment with PMA provides a positive control for the activation of MAPk in many cell types ***(13)***.

Ultimately it is important to determine whether the active MAPk is being translocated into the nucleus where many of its critical substrates reside ***(14)***. This can be accomplished through confocal indirect immunofluorescence. Confocal localization of total MAPk confirms whether a significant fraction of cellular MAPk is relocalized to the nucleus following stimulation. It also indicates whether appearance of active MAPk in the nucleus is more likely to be owing to phosphorylation of MAPk in the cytosol and its subsequent translocation to the nucleus, or whether there may be preexisting inactive MAPk in the nucleus that may be phosphorylated and activated *in situ*. The former paradigm apparently operates in SKNSH cells (*see* data figures later).

3.1. Preparation of Samples for Assay of Active MAPk by Western Blotting

1. The wild-type and drug-resistant SKNSH human neuroblastoma cells are passaged when approaching confluence with trypsin/EDTA to provide new maintenance cultures on 100-mm tissue dishes and experimental cultures on 35-mm dishes. One 35-mm dish is required for each experimental data point. A 1:40 split of the wild-type cells and a 1:20 split of the resistant cells will provide experimental cultures that are approaching confluence after 48 h. At this point the cultures are rinsed twice with DMEM (without serum) and incubated for a further 24 h in DMEM (without serum) (*see* **Note 6**).
2. All the materials required for the treatment and termination protocol are made ready: the agonists at appropriate stock concentration for 1:1000 dilution into the

cultures; a labeled microcentrifuge tube for each sample with a hole poked in the cap using a 26-gauge syringe needle; a vacuum aspirator; a hot-block at 100ºC; cell lysis buffer heated to 100ºC.
3. The cultures are treated with agonists according to the protocol and the medium then removed by aspiration. Immediately, 100 µL of boiling cell lysis buffer is added and the material scraped into the appropriate labeled tube (*see* **Note 7**).
4. The tubes are closed and then boiled for a further 10 min. After cooling to room temperature, they are ready for separation by SDS-PAGE.

3.2. SDS-PAGE

1. These instructions assume the use of a Hoeffer SE-400 or SE-600 gel system. They are easily adaptable to other formats, including minigels. It is critical that the glass plates for the gels are scrubbed clean with a rinsable detergent after use (e.g., Alconox, Alconox, New York, NY) and rinsed extensively with distilled water. They can be kept clean until use in a plastic rack in 30% nitric acid (use caution when removing). They will just need rinsing (distilled water then 95% ethanol) to remove the acid and air-dry.
2. Prepare a 1.5-mm thick, 10% gel by mixing 7.5 mL of 4X separating buffer, with 10-mL acrylamide/bis solution, 12.5 mL water, 100 µL ammonium persulfate solution, and 20 µL TEMED. Pour the gel, leaving space for a stacking gel, and overlay with water-saturated isobutanol. The gel should polymerize in about 30 min.
3. Pour off the isobutanol and rinse the top of the gel twice with water.
4. Prepare the stacking gel by mixing 2.5 mL of 4X stacking buffer with 1.3 mL acrylamide/*bis* solution, 6.1 mL water, 50 µL ammonium persulfate solution, and 10 µL TEMED. Use about 0.5 mL of this to quickly rinse the top of the gel and then pour the stack and insert the comb. The stacking gel should polymerize within 30 min.
5. Prepare the running buffer by diluting 100 mL of the 4X running buffer with 400 mL of water in a measuring cylinder. Cover with Para-Film and invert to mix.
6. Once the stacking gel has set, carefully remove the comb and use a 3-mL syringe fitted with a 22-gauge needle to wash the wells with running buffer.
7. Add the running buffer to the upper and lower chambers of the gel unit and load the 50 µL of each sample in a well. Include one well for prestained molecular weight markers.
8. Complete the assembly of the gel unit and connect to a power supply. The gel can be run either overnight at 50 V or, if cooling is available for the gel unit, then during the day (about 5 h) at 20 mA through the stacking gel and 30–40 mA through the separating gel. The dye fronts (blue and pink) can be run off the gel if desired, but if the pink dye (pyronin-Y) is retained then it will be transferred to the nitrocellulose membrane and identify the positions of the lanes.

3.3. Western Blotting for Active MAPk

1. The samples that have been separated by SDS-page are transferred to supported nitrocellulose membranes electrophoretically. These directions assume the use of a

Hoeffer transfer tank system. A tray of setup buffer is prepared that is large enough to lay out a transfer cassette with its pieces of foam and with two sheets of 3MM paper submerged on one side. A sheet of the nitrocellulose cut just larger than the size of the separating gel is laid on the surface of a separate tray of distilled water to allow the membrane to wet by capillary action. The membrane is then submerged in the setup buffer on top of the 3MM paper.

2. The gel unit is disconnected from the power supply and disassembled. The stacking gel is removed and discarded and one corner cut from the separating gel to allow its orientation to be tracked. The separating gel is then laid on top of the nitrocellulose membrane.
3. Two further sheets of 3MM paper are wetted in the setup buffer and carefully laid on top of the gel, ensuring that no bubbles are trapped in the resulting sandwich. The second wet foam sheet is laid on top and the transfer cassette closed.
4. The cassette is placed into the transfer tank such that the nitrocellulose membrane is between the gel and the anode. It is vitally important to ensure this orientation or the proteins will be lost from the gel into the buffer rather than transferred to the nitrocellulose.
5. The refrigerated/circulating water bath is switched on to maintain a temperature between 10–15ºC and a magnetic stir-bar in the tank activated.
6. The lid is put on the tank and the power supply activated. Transfers can be accomplished at either 30 V overnight or 70 V for 2 h.
7. Once the transfer is complete the cassette is taken out of the tank and carefully disassembled, with the top sponge and sheets of 3MM paper removed. The gel is left in place on top of the nitrocellulose and these are laid on a glass plate so that the shape of the gel (including the cut corner for orientation) can be cut into the membrane using a razor blade. The gel and excess nitrocellulose can then be discarded. The coloured molecular weight markers (and pyronin-Y lane markers, if the latter were retained on the gel during the PAGE run) should be clearly visible on the membrane.
8. The nitrocellulose is then incubated in 50 mL blocking buffer for 1 h at room temperature on a rocking platform.
9. The blocking buffer is discarded and the membrane quickly rinsed prior to addition of a 1:2000 dilution of the antidually phosphorylated MAPk antibody in TBS-T/2% BSA for 1 h at room temperature on a rocking platform.
10. The primary antibody is then removed (*see* **Note 8**) and the membrane washed three times for 5 min each with 50 mL TBS-T.
11. The secondary antibody is freshly prepared for each experiment as 1:20,000-fold dilution in blocking buffer and added to the membrane for 30 min at room temperature on a rocking platform.
12. The secondary antibody is discarded and the membrane washed six times for 10 min each with TBS-T.
13. During the final wash, 2 mL aliquots of each portion of the ECL reagent are warmed separately to room temperature and the remaining steps are done in a dark room under safe light conditions. Once the final wash is removed from the blot, the ECL

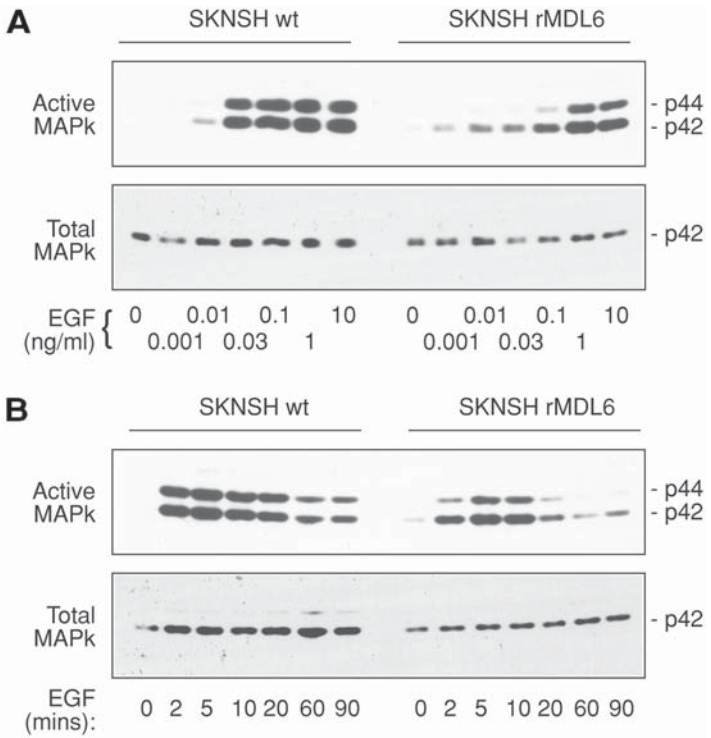

Fig. 1. Concentration-response and kinetics of the activation of MAPk by EGF in SKNSH rMDL6 cells. (**A**) Wild-type and SKNSH rMDL6 cells were treated for 5 min with the indicated concentrations of EGF and processed for assay of active and total MAPk by western blotting. (**B**) Wild-type and SKNSH rMDL6 cells were treated for the indicated time with 10 ng/mL EGF and processed for assay of active and total MAPk by western blotting. Activation of MAPk by EGF is clearly less potent and more transient in rMDL6 cells than in wild-type SKNSH cells. EGF, even up to 175 ng/mL, was incapable of activation of MAPk in rDOX6 cells (data not shown). (Reproduced from **ref. *9*** with permission from Elsevier Science.)

reagents are mixed together and then immediately added to the blot, which is then rotated by hand for 1 min to ensure even coverage.

14. The blot is removed from the ECL reagents, blotted with Kim-Wipes, and then placed between the leaves of an acetate sheet protector that has been cut to the size of an X-ray film cassette (*see* **Note 9**).
15. The acetate containing the membrane is then placed in an X-ray film cassette with film for a suitable exposure time, typically a few minutes. An example of the results produced is shown in **Fig. 1**.

3.4. Stripping and Reprobing Blots for Total MAPk

1. Once a satisfactory exposure for the result of the active MAPk has been obtained, the membrane is then stripped of that signal and then reprobed with an antibody that recognizes MAPk irrespective of whether it is phosphorylated. This provides a loading control that confirms equal recovery of the samples through the procedure.
2. Stripping buffer (50 mL per blot—*see* **Note 10**) is warmed to 70ºC and the β-mercaptoethanol and blot added. The blot is incubated for 30 min with occasional agitation.
3. Once the blot is stripped it is extensively washed in washing buffer (three times 150 mL, each wash for 10 min), and then blocked again in blocking buffer.
4. The membrane is then ready to be reprobed with anti-total MAPk (1:5000 in TBS-T/2% BSA) with washes, secondary antibody, and ECL detection as above. An example result is shown in **Fig. 1**.

3.5. Confocal Immunofluorescence for Active and Total MAPk

1. Neuroblastoma cells are passaged as described above (***see*** **Subheading 3.1.**), except that the experimental samples are either 60-mm dishes with sterile cover-slips or two-well chamber slides. If cover-slips are to be used, they must first be sterilized by holding with tweezers, addition of 95% ethanol, and passing through the flame of a Bunsen burner (take extra care to keep the flame away from the ethanol bottle and not to allow the burning ethanol to drip onto a flammable surface), and then placed in the culture dishes to cool.
2. The cells should be rinsed and changed to serum-free DMEM when below subconfluence (typically, 24–48 h of culture time) so that individual cells are clearly visible in the immunofluorescence.
3. The cells are treated with agonists according to the protocol, and then rinsed rapidly twice with ice-cold PBS.
4. Paraformaldehyde solution is then added for 10 min at room temperature to fix the cells.
5. The paraformaldehyde is discarded (into a hazardous waste container) and the samples washed twice for 5 min each with PBS.
6. Residual formaldehyde is quenched by incubation in NH_4Cl for 10 min at room temperature, followed by a further two washes with PBS.
7. The cells are permeabilized by incubation in PBS/Triton X-100 for 5 min at room temperature, and then rinsed twice more with PBS.
8. The samples are blocked by incubation in antibody dilution buffer for either 2 h at room temperature or at 4ºC overnight. If chamber slides are used, then the upper plastic housing should be removed, leaving the gasket on the slide.
9. The blocking solution is removed and replaced with the antiactive MAPk antibody (1:200) or antitotal MAPk (1:50) in antibody dilution buffer for 1 h at room temperature (*see* **Note 11**).
10. The primary antibody is removed and the sample washed three times for 5 min each with PBS. The sample is then put under aluminium foil and the room lights dimmed for subsequent steps.

11. The secondary antibody is prepared at 1:250 in antibody dilution buffer and added to the samples for 30 min at room temperature (*see* **Note 11**).
12. The secondary antibody is discarded and DAPI is added for 10 min at room temperature to stain the DNA and identify the nuclei.
13. The samples are washed five times for 10 min each with PBS and then aspirated dry from one corner.
14. The samples are then ready to be mounted. If they are on a cover-slip then the slip is carefully inverted into a drop of mounting medium on a microscope slide. If on a chamber slide, then the gasket is carefully removed (a razor blade may be required) and then mounting medium and a cover-slip added. In either case, nail varnish is used to seal the sample (*see* **Note 12**). The sample can be viewed immediately that the varnish is dry, or be stored in the dark at 4ºC for up to a month.
15. The slides are viewed under phase contrast microscopy (to locate the cells and identify the focal plane) and under confocal microscopy. Excitation at 543 nm induces the Cy3 fluorescence (red emission) for the MAPk, while excitation at 364 nm induces DAPI fluorescence (blue emission). Software can be used to overlay the phase contrast and fluorescence images. Examples of the signals for active MAPK and total MAPk are shown in **Figs. 2** and **3**.

4. Notes

1. Unless stated otherwise, all solutions should be prepared in water that has a resistivity of 18.2 MΩ-cm and total organic content of less than five parts per billion. This standard is referred to as "water" in this text.
2. TEMED is best stored at room temperature in a desiccator. Buy small bottles as it may decline in quality (gels will take longer to polymerize) after opening.
3. Transfer buffer can be used for up to five transfers within 1 wk so long as the voltage is maintained constant for each successive run (the current will increase each time). Adequate cooling to keep the buffer no warmer than room temperature by use of a refrigerated/circulating bath is essential to prevent heat-induced damage to the apparatus and the experiment.
4. We have found this antibody to be excellent for both Western blotting and immunofluorescence. Numerous competitive reagents are available from other commercial sources.
5. Quantification of data may be desired and this can be done by scanning densitometry of the films, providing that care is taken to ensure that the signal has not saturated. Alternatively, the chemiluminescent signal can be captured digitally with an instrument such as a FujiFilm LAS-1000 plus.
6. This protocol can be adapted for many other cell culture systems. For cells that grow in suspension, such as Jurkat T cells, the cells can easily be counted after the serum starvation and then aliquotted for the treatment protocol ***(13)***.
7. The cell material will be very viscous at this stage owing to release of DNA and thus hard to pipet. It is easiest to use the pipet tip to transfer the sample to the tube by dragging and pushing rather than by drawing the sample up and down. The viscosity

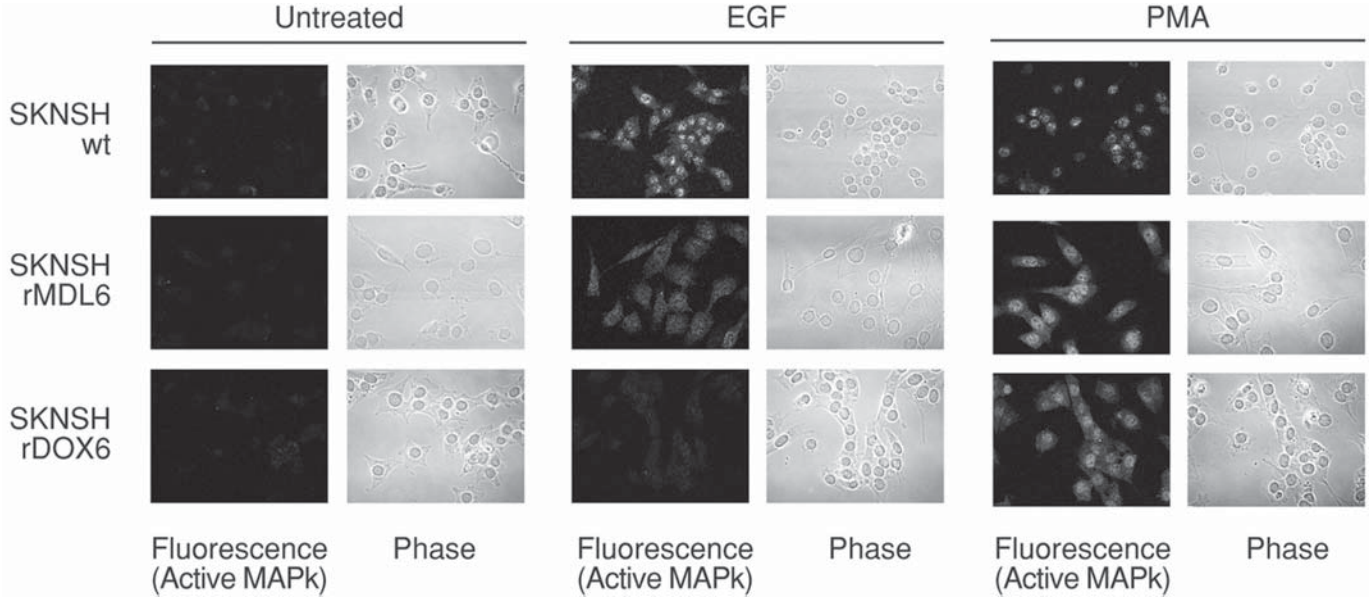

Fig. 2. Accumulation of active MAPk in the nuclei of control and stimulated SKNSH cells and drug-resistant variants. Wild-type SKNSH and the rMDL6 and rDOX6 variants were treated for 5 min with 10 ng/mL EGF or 200 n*M* PMA and processed for immunofluorescence of active MAPk. Confocal images were obtained on a Zeiss LSM310 microscope using a 63X oil-immersion lens as described ***(15)***. Phase contrast pictures of the same field of cells at the same magnification are also shown. The apparent nuclear localization that is evident following agonist stimulation of the wild-type cells was confirmed by colocalization with the fluorescence from DAPI, a DNA marker (data not shown). EGF is evidently better able to induce nuclear accumulation of active MAPk in wild-type SKNSH cells than it is in the resistant lines. PMA stimulates some active MAPk accumulation in the resistant lines, although, again it appears less effective than in the wild-type SKNSH cells. (Reproduced from **ref. *9*** with permission from Elsevier Science.)

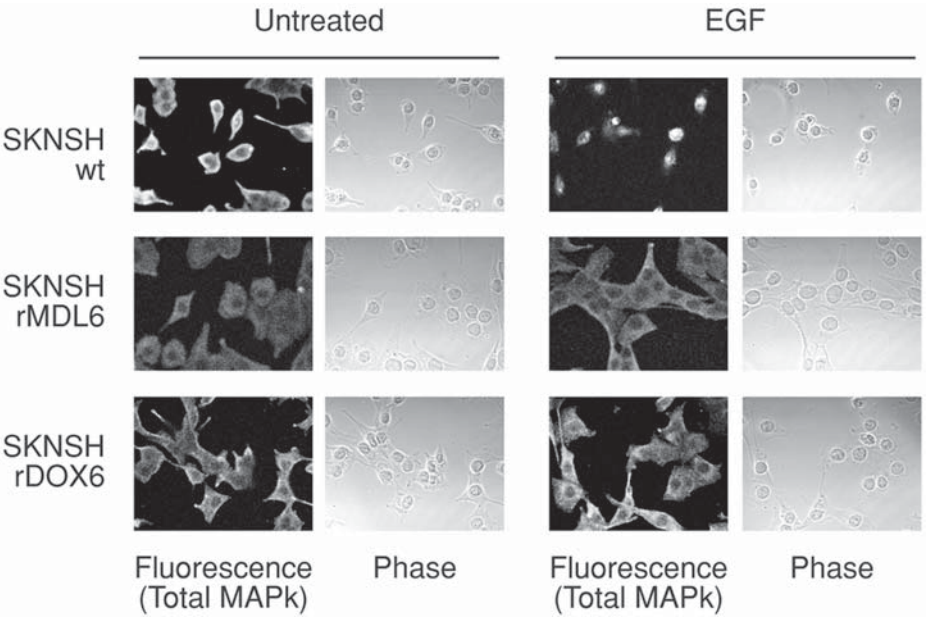

Fig. 3. EGF stimulation of nuclear translocation of MAPk in SKNSH cells. Wild-type SKNSH and the rMDL6 and rDOX6 variants were treated for 10 min with 10 ng/mL EGF and processed for confocal immunofluorescence of total MAPk. Phase contrast pictures of the same field of cells at the same magnification are also shown. The apparent nuclear localization that is evident following agonist stimulation of the wild-type cells was confirmed by colocalization with the fluorescence from DAPI, a DNA marker (data not shown). These results support a model whereby EGF induces the translocation of active MAPk from the cytosol to the nucleus of wild-type SKNSH cells, but is much less effective in the resistant lines. (Reproduced from **ref. *9*** with permission from Elsevier Science.)

of the material will decrease during subsequent boiling, allowing accurate loading of the gel.

8. The primary antibody can be saved for subsequent experiments by addition of 0.02% final concentration sodium azide (conveniently done by dilution from a 10% stock solution; exercise caution since azide is highly toxic) and storage at 4ºC. These primary antibodies have been used for up to 20 blots over several months, with the only adjustment required being increasing length of exposure to film at the ECL step.
9. Backgrounds in this protocol are normally so clean that exact alignment of the subsequent film with the nitrocellulose can be difficult. We, therefore, apply a square of luminescent tape (Sigma) to the edge of the acetate sheet to provide an alignment

mark for the film and membrane and thus allow identification of the signals with the lanes.

10. This procedure generates a significant, unpleasant smell in the laboratory. We, therefore, routinely wait until several membranes are ready to be stripped and process them in a group to minimize the time required. Containers with tight-fitting lids are an advantage. The use of a supported nitrocellulose membrane, rather than pure nitrocellulose, facilitates the increased manipulation required for stripping and reprobing.
11. For economy, only 100–150 µL of diluted antibody per sample needs to be used at this step. In the chamber slides, this volume will be retained by the gasket around the sample. If using cover-slips, then use an aspirator to dry the dish around the slip thoroughly and establish a good meniscus on top of the cover-slip with this volume of antibody dilution buffer before replacing it with the primary antibody.
12. Air bubbles are undesirable in the mounting medium, and slow, careful application of the top layer minimizes their appearance. A bright color of varnish is easier to apply accurately in the dark, with two thin coats being preferable.

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References

1. Guan, K. L. (1994) The mitogen activated protein kinase signal transduction pathway: from the cell surface to the nucleus. *Cell Signal.* **6,** 581–589.
2. Seger, R. and Krebs, E. G. (1995) The MAPK signaling cascade. *FASEB J.* **9,** 726–735.
3. Oka, H., Chatani, Y., Hoshino, R., Ogawa, O., Kakehi, Y., Terachi, T., et al. (1985) Constitutive activation of mitogen-activated (MAP) kinases in human renal cell carcinoma. *Cancer Res.* **55,** 4182–4187.
4. Krueger, J. S., Keshamouni, V. G., Atanaskova, N., and Reddy, K. B. (2001) Temporal and quantitative regulation of mitogen-activated protein kinase (MAPK) modulates cell motility and invasion. *Oncogene* **20,** 4209–4218.
5. Sebolt-Leopold, J. S., Dudley, D. T., Herrera, R., Van Becelaere, K., Wiland, A., Gowan, R. C., et al. (1999) Blockade of the MAP kinase pathway suppresses growth of colon tumors in vivo. *Nature Med.* **5,** 810–816.
6. Nishio, K., Nakamura, T., Koh, Y., Suzuki, T., Fukumoto, H., and Saijo, N. (1999) Drug resistance in lung cancer. *Curr. Opinion Oncol.* **11,** 109–115.
7. Tewey, K. M., Rowe, T. C., Yang, L., Halligan, B. D., and Liu, L. F. (1984) Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II. *Science* **226,** 466–468.

8. Mirkin, B. L., O'Dea, R. F., and Hogenkamp, H. P. (1987) Cytotoxic action of adenosine nucleoside and dialdehyde analogues on murine neuroblastoma in tissue culture. *Cancer Res.* **47,** 3650–3655.
9. Mattingly, R. R., Milstein, M. L., and Mirkin, B. L. (2001) Down-regulation of growth factor-stimulated MAP kinase signaling in cytotoxic drug-resistant human neuroblastoma cells. *Cell Signal.* **13,** 499–505.
10. Ray, L. B. and Sturgill, T. W. (1988) Insulin-stimulated microtubule-associated protein kinase is phosphorylated on tyrosine and threonine in vivo. *Proc. Natl. Acad. Sci. USA* **85,** 3753–3757.
11. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.
12. Gabay, L., Seger, R., and Shilo, B.-Z. (1997) In situ activation pattern of Drosophila EGF receptor pathway during development. *Science* **277,** 1103–1106.
13. Mattingly, R. R., Felczak, A, Chen, C.-C., McCabe, M. J. Jr., and Rosenspire, A. J. (2001) Low concentrations of inorganic mercury inhibit Ras activation during T cell receptor mediated signal transduction. *Toxciol. Appl. Pharmacol.* **176,** 162–168.
14. Chen, R. H., Sarnecki, C., and Blenis, J. (1992) Nuclear localization and regulation of erk- and rsk-encoded protein kinases. *Mol. Cell. Biol.* **12,** 915–927.
15. Mattingly, R. R., Saini, V., and Macara, I. G. (1999) Activation of the Ras-GRF/CDC25Mm exchange factor by lysophosphatidic acid. *Cell Signal.* **11,** 603–610.

7

TUNEL and Immunofluorescence Double-Labeling Assay for Apoptotic Cells with Specific Antigen(s)

Stephanie M. Oberhaus

Abstract

A double-labeling assay for detecting apoptotic cells, using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) assay, and antigens of interest, using immunofluorescence, is described. The assay has been used successfully on fixed, cultured cells and formalin-fixed paraffin-embedded tissue sections. This *in situ* detection system can be used to correlate apoptosis with specific antigen expression to identify factors involved in the induction and execution of the apoptotic pathway of cell death in a wide variety of experimental systems.

Key Words: Apoptosis; TUNEL; DNA fragmentation; immunofluorescence; reovirus.

1. Introduction

The ability to identify and quantitate apoptotic cells and to correlate apoptosis with specific antigen expression is a powerful tool for studying the apoptotic process in vitro and in vivo. The detection of DNA fragmentation associated with apoptotic cells using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) assay has been the most widely used assay for identifying apoptotic cells *in situ*. In this assay, biotinylated dUTP is incorporated at the 3' OH ends of fragmented DNA using TdT, and detected by a variety of methods involving brightfield or fluorescence microscopy ***(1)***. The labeling of fragmented DNA also facilitates detection of condensed chromatin, another characteristic morphological change associated with apoptotic cells ***(2)***. The localization of the staining to the nuclear DNA allows for additional detection

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of cytoplasmic antigens of interest. Because DNA fragmentation can also be associated with necrotic cell death, it is important to perform at least one additional assay for apoptosis based on a different criterion, e.g., caspase activation or phosphatidylserine exposure (and so on) ***(3)***. The TUNEL assay is not a cost-effective method for screening large numbers of samples, because it costs nearly $70 per slide for the biotinylated dUTP and TdT alone. However, TUNEL, especially in conjunction with colabeling for specific antigens, is a powerful assay for characterizing the apoptotic process *in situ*. The choice of antigens for detection and the antibodies used to detect them should be considered carefully because many proteins are cleaved during apoptosis and may be modified antigenically. We have used the double-labeling assay described here to characterize reovirus-induced apoptosis in vivo and in a variety of infected cancer cell lines in an effort to understand the mechanism(s) involved in viral-induced cell death of various cell types ***(4)***.

2. Materials

2.1. Solutions and Reagents

1. High-quality distilled, deionized water (DDW) should be used in preparing solutions and rinsing glassware, and for washing steps.
2. Acetone stored at –20ºC.
3. 0.01 *M* citrate buffer, pH 6.0: mix 9.5 mL of 0.1 *M* citric acid + 41.5 mL of 0.1 *M* sodium citrate + 949 mL DDW, stored as a sterile solution and used at room temperature.
4. Phosphate-buffered saline (PBS) stored as a sterile solution and used at room temperature.
5. Proteinase K digestion mix: 5 µg/mL in 10 m*M* Tris-HCl, (pH 7.5), 2 m*M* $CaCl_2$ in DDW (proteinase K stock should be made at 10 mg/mL in DDW, filtered, and stored in small aliquots at –20ºC to avoid repeated freezing and thawing).
6. Hydrogen peroxide, generally sold as 30% solution, stored at 4ºC.
7. Terminal deoxynucleotidyl transferase (TdT) buffer: 30 m*M* Tris-HCl, pH 7.2, 140 m*M* cacodylic acid (sodium cacodylate trihydrate, SigmaUltra) (Sigma, St. Louis, MO, suspected carcinogen), 1 m*M* cobalt chloride (cobalt II chloride hexahydrate, ACS reagent) (Sigma, harmful by inhalation or skin contact, possible mutagen).
8. Biotin-16-dUTP (Roche Applied Science, Indianapolis, IN), stored at –20ºC.
9. Terminal deoxynucleotidyl transferase (TdT) (Invitrogen, Carlsbad, CA) stored at –20ºC.
10. Vectastain Elite Peroxidase ABC staining kit (Vector Laboratories, Burlingame, CA) stored at 4ºC.
11. 4X SSC buffer: 0.6 *M* NaCl, 0.06 *M* sodium citrate (pH 7.0) stored as a sterile solution and used at room temperature.
12. Bovine serum albumin (BSA) (minimum 96%, electrophoresis-grade, pH 7.0, lyophilized powder [Sigma] stored as powder at 4ºC), 2% solution in DDW made before use.

13. DAB kit (Vector Laboratories) stored at 4ºC or DAB stock (60 mg 3, 3' diaminobenzidene (Sigma) dissolved in 200 mL PBS and stored frozen in 5-mL aliquots, protected from light; DAB is a suspected carcinogen.
14. Blocking solution: 0.2% Triton X-100, 2% normal goat serum (or species of origin of secondary antibody used for immunofluorescence), 1% BSA in PBS stored as a nonsterile solution at 4ºC for approx 1 mo.
15. Primary antibody of interest, e.g., antireovirus polyclonal antiserum.
16. Secondary antibody, e.g., cyanine-2 (Cy-2)-conjugated goat anti-Rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA).
17. Mounting medium (Aqua Polymount, Polysciences, Inc., Warrington, PA) stored at 4ºC upside down to prevent air bubbles when dispensing.
18. 5% dimethylchlorosilane (Sigma) in chloroform (made just before use).

2.2. Equipment and Consumables

1. Chambered slides (Nunc Lab-Tek II).
2. Staining jars (Fisher Scientific) (*see* **Note 1**).
3. Large staining containers.
4. Slide mailers (*see* **Note 2**).
5. Forceps for handling slides.
6. Humidified chamber: small plastic storage container with tight-fitting lid, and platform to hold slides above wetted paper towels. Cut paper towels to lie flat on bottom of container, place platform (the tip holder removed from pipet tip boxes works well) on top of paper towels and press on towels until level. Slides will be placed on platform.
7. Cover slips: silanized (for incubating with reaction mixes) and nonsilanized (for mounting) (22 × 50 mm, No.1 for LabTeks, 24 × 30 or 40 mm, No. 1 for tissue sections) (*see* **Note 3**).

3. Methods

3.1. Silanizing Cover Slips

In a fume hood (vapors are toxic), prepare silane solution (*see* **Subheading 2.1., item 18.**) in a small, tall glass beaker. Using forceps, dip cover slips in the solution so they are completely coated (may have to flip), then stand them vertically to dry (against the posts of a multipost test tube rack). Rinse cover slips by dipping them one-by-one in a 2-L beaker of DDW or place in a glass Petri dish and rinse at least 10 times with DDW; stand vertically to dry; bake at 180ºC overnight, and store in a Petri dish. Dispose of silane solution appropriately.

3.2. Initial Steps of TUNEL Assay for Cultured, Adherent Cells

3.2.1. Plating the Cells and Inducing Apoptosis

1. Plate cells at desired concentration (e.g., 6×10^4 cells/well in 300 µL of medium for an eight-chambered Lab Tek slide).

2. Allow cells to adhere (usually overnight), then treat with inducer(s) of apoptosis (e.g., virus infection, serum deprivation). Include a negative control (e.g., mock infection, no serum deprivation) and a positive control (treatment with a known inducer of apoptosis) for initial assay development (*see* **Note 4**).
3. Allow cells to incubate for desired time-point(s); this may vary from hours to days, depending on the cells and treatments.

For all of the following steps, work quickly and do not allow the cell monolayers to dry out, or both TUNEL and immunofluorescence artifacts can occur.

3.2.2. Fixing the Cells

1. Gently aspirate the medium.
2. Gently fill wells with PBS to rinse medium and aspirate.
3. Gently add cold acetone to wells (just enough to cover cell monolayers).
4. Incubate at 4ºC, 10 min, then aspirate.
5. Fill wells with PBS and remove chambers using the tool included with the slides.
6. Transfer slide(s) to staining jar with PBS (enough to cover slides), 5 min.
7. Transfer slide(s) to staining jar with PBS (enough to cover slides), 5 min, or they can be stored this way at 4ºC indefinitely (cover top with the cap to prevent evaporation).

3.2.3. Optional Microwave Treatment

We have found that this microwave treatment reduces background in the TUNEL assay when performed on fixed cells, but not with tissue sections ***(5)***. All steps are performed at room temperature, unless noted otherwise. This treatment may also enhance the antigenicity of some antigens (*see* **Note 5**).

1. Place slides in 200 mL 0.01 *M* citrate buffer, pH 6.0.
2. Microwave at highest setting just until it boils.
3. Quickly transfer slides to staining jar with cold (4ºC) PBS, 5 min.

3.3. Initial Steps of TUNEL Assay for Tissue Sections from Formalin-Fixed Paraffin-Embedded Tissue Blocks

Experiments with animals should include a negative control (e.g., mock infection, no treatment) and a positive control (treatment with a known inducer of apoptosis) for initial assay development (*see* **Note 4**). Digestion with proteinase K can enhance the sensitivity of the TUNEL assay with fixed tissues, and the antigenicity of some, but not all antigens of interest. The benefits, if any, and the optimal digestion conditions must be determined for each antigen of interest (*see* **Note 6**). The following conditions work well for detecting TUNEL-positive and reovirus-infected cells in tissue sections from formalin-fixed liver and brain and paraformaldehyde-fixed brain from neonatal mice ***(4)***. For all of the

following steps, work quickly and do not allow the tissue sections to dry out, or both TUNEL and immunofluorescence artifacts can occur.

1. Tissue sections (3–5 per slide, 5- to 10-µm thick, depending on the tissue) should be deparaffinized and rehydrated according to standard protocols ***(6)***.
2. Incubate slides in proteinase K digestion mix (*see* **Subheading 2.1., item 5.**) in a mailer for 30 min at 37ºC.
3. Rinse slides in PBS, 2 × 5 min.
4. Proceed to the TUNEL assay (*see* **Subheading 3.4., step 1.**).

3.4. TUNEL Assay

All steps should be performed at room temperature, unless noted otherwise. All incubations and washes should be performed in staining jars, unless noted otherwise.

1. Incubate slides in 0.3% H_2O_2 in PBS, 15 min to inactivate endogenous peroxidase (ex. 400 µL 30% stock + 40 mL PBS) (*see* **Note 7**).
2. Rinse slides in DDW: 2 × 5 min.
3. Incubate slides in TdT buffer (*see* **Subheading 2.1., item 7.**), 5 min.
4. Prepare TdT reaction mix (approx 150 µL/LabTek chambered slide, approx 75 µL/slides with tissue sections): 50 nmol of biotin-16-dUTP/mL and 500-750U of TdT/mL in TdT buffer supplied with enzyme (e.g., 105 µL DDW, 30 µL 5X TdT buffer, 7.5 µL biotin-16-dUTP, 7.5 µL TdT).
5. Remove slide from TdT buffer and flick to remove excess liquid, dry back of slide, and lay flat on the platform in the humidified chamber. Use a pipettor to layer approx 150 or approx 75 µL of TdT reaction mix evenly over cell monolayer or tissue section, respectively. Gently cover with a silanized cover slip to ensure even coverage. Gently tap the top of the cover slip to remove large air bubbles. Snap lid on container and incubate at 37ºC, 1 h.
6. Just before the end of the 1-h incubation period, prepare the avidin-biotin-peroxidase complex (ABC) solution in a 15-mL tube: 14 mL PBS + 63 µL "A" + 63 µL "B" (*see* **Note 8**). Mix on a Nutator or similar mixing apparatus for at least 30 min.
7. Remove slides from the humidified chamber and hold vertically to allow cover slips to slide off. If a cover slip sticks, submerge slide in 4X SSC (next step) and pull slide out. (The cover slip should slide off and can be removed from the jar.)
8. Stop the TdT reaction by incubating slides in 4X SSC, 15 min.
9. Rinse slides in DDW: 2 × 5 min.
10. Flick slides to remove excess liquid, dry back of slides, and lay flat on a piece of Parafilm. Using a pipet, gently layer 2% BSA solution over slide to completely cover the slide. Incubate 10 min (*see* **Note 9**).
11. Wash slides in PBS: 2 × 5 min.
12. Incubate slides in ABC solution in a mailer, 1 h.
13. Wash slides in PBS: 3 × 10 min.

14. Develop with DAB substrate (kit or own reagents, *see* **Subheading 2.1.13.**):
    a. **DAB kit:** Add to 15 mL DDW, in this order, mixing after each addition: 6 drops buffer, 12 drops DAB solution, 6 drops H_2O_2 (*see* **Note 10**).
    b. **DAB stock:** Thaw three 5-mL aliquots just before use, pool in a test tube, add H_2O_2 to 0.1%, and filter through 0.2-µm syringe top filter.

    Transfer DAB developing solution to a mailer. Remove slides from PBS, flick to remove excess liquid, and transfer to mailer with DAB. Check development of color every 30 to 60 s; development can be stopped reversibly by transferring slides to PBS, then returning to DAB solution for further development (*see* **Note 11**). When color development is complete, wash slides in PBS, 2 × 5 min. (*see* **Note 12**).
15. Cover slips may be mounted at this time with mounting solution (Aqua PolyMount) (*see* **Subheading 3.5., step 6.**) or slides may be processed for immunofluorescence (*see* **Note 13**).

3.5. Immunofluorescence Staining

1. Incubate slides in blocking solution (*see* **Subheading 2.1.14**) in a mailer for at least 30 min at room temperature (longer is fine, even overnight at 4ºC).
2. Incubate slides with primary antibody diluted in blocking solution (dilution will have to be determined for optimal staining). Remove slides from blocking solution and flick to remove excess liquid. Dry back of slides and lay on platform of humidified chamber. Layer primary antibody solution over cells to cover (as described for TdT reaction mix, *see* **Subheading 3.4., items 4 and 5**). Optimal incubation conditions will vary for different antibodies. For detection of reovirus antigens using polyclonal antiserum (1:200), we incubate at room temperature in the humidified cham-ber for several hours and then at 4ºC overnight *(7)*.
3. Remove slides from humidified chamber and hold vertically to allow cover slips and excess liquid to come off. Wash slides in PBS, 3 × 10 min.
4. Incubate with secondary antibody diluted in blocking solution (ex. 1:50 for Cy-2-goat antirabbit serum). Remove slides from PBS and flick to remove excess liquid. Dry back of slides and lay on platform of humidified chamber. Layer secondary antibody solution over cells (as described for TdT reaction mix, *see* **Subheading 3.4., items 4 and 5**). Wrap humidified chamber in foil to protect from light and incubate at 37ºC for 1 h.
5. Warm mounting solution by transferring it from 4ºC to room temperature. Remove slides from humidified chamber and hold vertically to allow cover slips and excess liquid to come off. Wash slides in PBS, protected from light, 3 × 10 min.
6. Flick slides vigorously to remove excess moisture, and mount cover slips. Dispense mounting solution onto slide, approx 1 drop/well of LabTek chambered slide or make a "T" lengthwise down slide over tissue sections. Lower nonsilanized cover slip at an angle over mounting solution to avoid air bubbles. If air bubbles form, they may be lightly tapped or pressed out using forceps. Allow slides to lay flat, protected from the light, overnight at 4ºC to allow the mounting solution to solidify (*see* **Note 13**).

7. Slides are best stored in slide holders, lying flat with cells or tissue sections facing up. For short-term storage (until images are taken) slides should be stored at 4ºC to prolong fluorescence intensity. For long-term storage, slides can be stored at room temperature.
8. Cy-2 fluorescence is relatively stable, but images should be taken as soon as possible for the strongest signal (*see* **Note 14**). TUNEL and fluorescence labeling can be viewed simultaneously by turning on the bright light while viewing fluorescence. The bright light can be adjusted using the intensity control and/or neutral density filters to optimize visualization of both fluorescence and DAB staining (*see* **Note 15**).

3.6. Analysis and Quantitation

Analysis of the TUNEL and immunofluorescence results can be done in a variety of ways. For cultured cells, four categories of cells can be counted: TUNEL-positive, antigen-positive, double-positive and unstained (*see* **Figs. 1** and **2**). Each of the positive cell types can then be expressed as a percentage of the total number of cells counted. To obtain statistically significant numbers, it is recommended that at least 100 cells/microscopic viewing field X 2–3 fields per sample is counted for duplicate samples. The means and standard error or deviation of the means can be calculated and compared using the student *t*-test, for example.

Quantitative analysis of results obtained for tissue sections can be more difficult to perform, and will likely vary with the tissue type and treatments. Apoptosis and antigen expression may also be correlated with tissue damage, localization within the tissue to specific structures (for example, bile ducts in the liver, hippocampus in the brain), specific cell types affected within the tissue, and other parameters. For example, reovirus-induced apoptosis in the livers of neonatal mice is usually localized to "patches" (defined as >5 cells) in and around portal triads, and involves specific cell types, including bile duct epithelial cells (*see* **Fig. 3**). In a case like this, simply counting the various categories of cells, as described for cultured cells, is not very informative.

For both cultured cells and tissue sections, a comparison of the results with appropriate negative controls is critical for assessing the effects of experimental treatments on the level of apoptosis and antigen expression.

4. Notes

1. These plastic staining (coplin) jars hold up to five slides (slides on the outside walls should face inward to avoid contact with the outside walls and damage to the cells/tissue sections). With five slides, 40 mL of solution is generally adequate to cover the samples on the slides; if fewer than five slides are processed, blank slides may be added to increase the height of the solution.

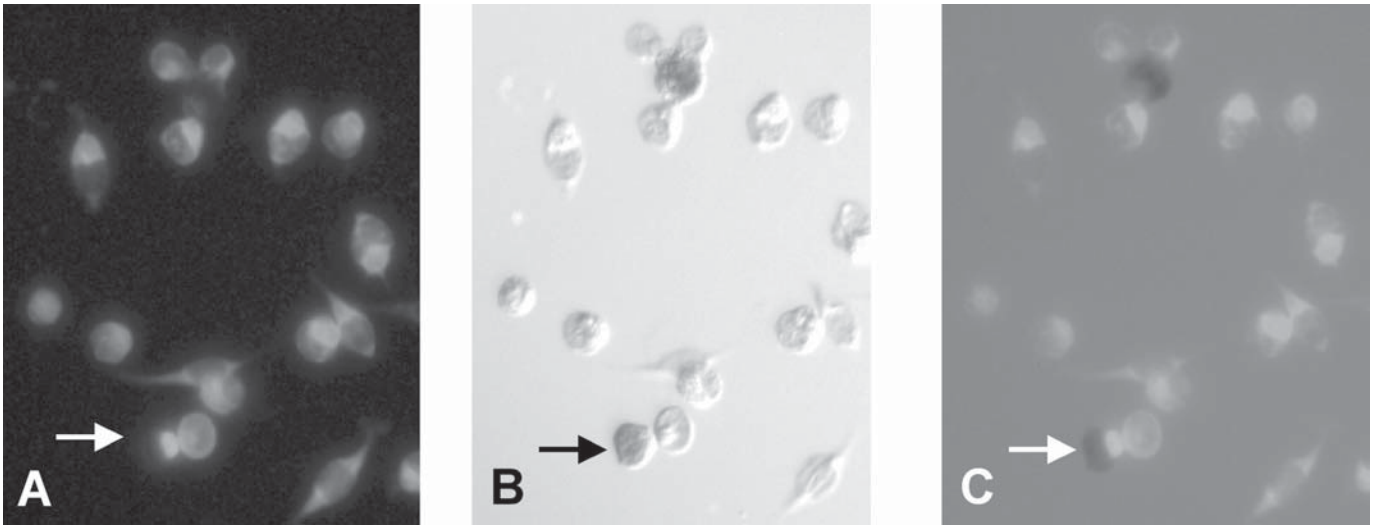

Fig. 1. Mouse fibroblasts (L cells) infected with reovirus strain T3 Abney. Cells were infected at a multiplicity of infection of 50 and the TUNEL/immunofluorescence assay was performed 48 h postinfection. The arrow points to a reovirus antigen-positive, TUNEL-positive cell in the same field viewed by (**A**) fluorescence, (**B**) brightfield (differential interference contrast), and (**C**) double exposure microscopy. A Nikon E600 epifluorescence microscope was used to view samples and digital images were saved using a Spot camera (Diagnostic Instruments, Inc.). Original magnification ×400.

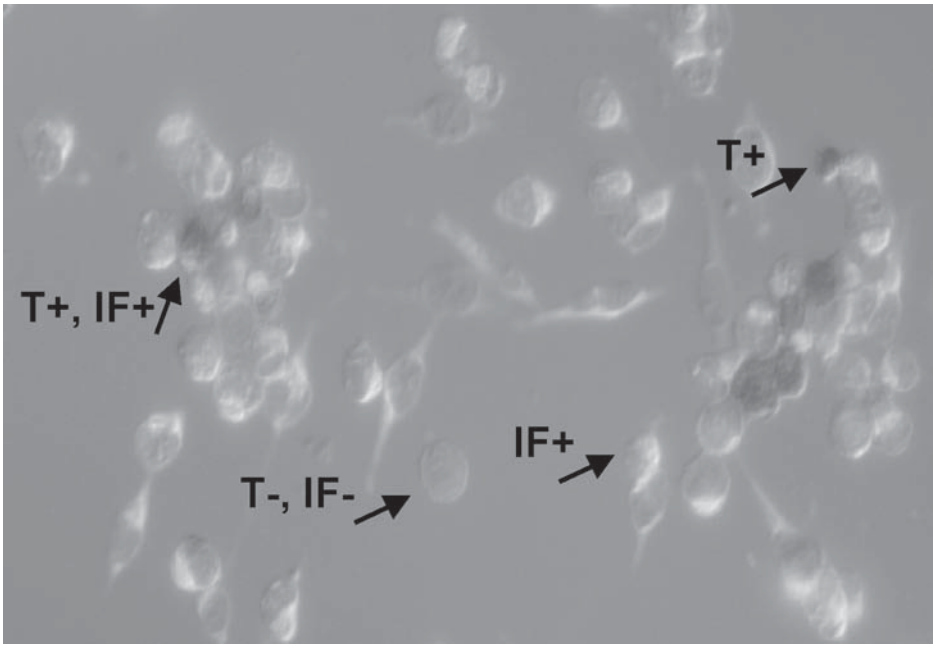

Fig. 2. Four categories of cells can be counted with the TUNEL (T)/immunofluorescence (IF) double labeling assay using double exposure microscopy: (T+, IF+), (T−, IF−), (IF+), and (T+), as shown here with mouse fibroblasts infected with reovirus strain T3A (described in **Fig. 1**). Original magnification ×400.

2. Each mailer holds five slides (like the staining jars) and 14 mL solution is more than enough to cover the samples on the slides. If fewer than five slides are processed, blank slides may be added to increase the height of the solution.
3. Silanized cover slips are recommended (but not always necessary) for ensuring coverage of samples with small volumes of reaction mixes and minimizing desiccation, because they do not stick easily to fixed cells or tissue sections.
4. Additional controls for the TUNEL assay include omission of the TdT enzyme during the TUNEL reaction (negative control) and treatment of fixed cells or tissue sections with DNase I to generate 3'OH ends in the DNA for labeling. These controls are excellent for testing the reaction conditions and reagents, but should not replace the positive and negative controls for the treatment conditions, nor be used to determine development times for the experimentally treated samples.
5. The benefits, if any, of microwave treatment on detection of TUNEL-positive and antigen-positive cells in fixed cultured cells or tissue sections must be determined for each sample type and antigen of interest. The conditions described work well for detecting TUNEL-positive and reovirus-infected cells in a variety of acetone-fixed cells. For example, mouse fibroblasts (L), breast cancer (MCF-7), mouse liver epithelial (NMuLi), human liver carcinoma (HepG2), and others.

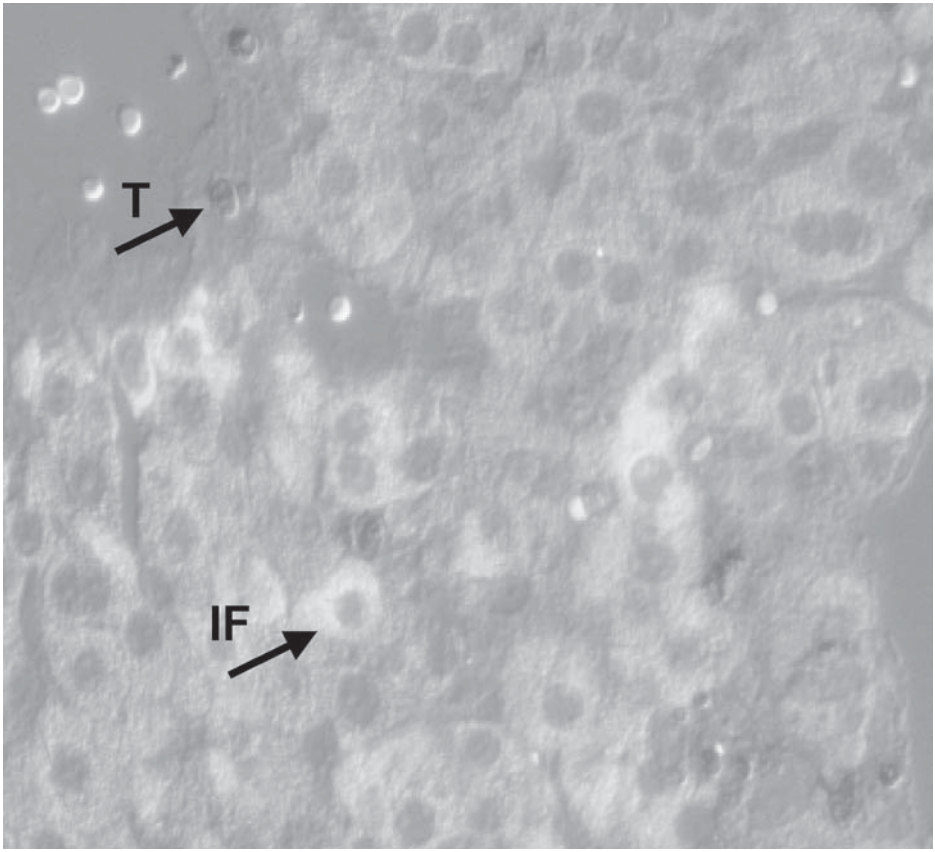

Fig. 3. TUNEL-positive (T) and reovirus-infected (immunofluorescence-positive, IF) cells detected in liver tissue from a neonatal mouse infected with reovirus strain T3 Abney, 6 d postinfection. Original magnification ×400.

6. The proteinase K digestion conditions, as described, cannot be used on fixed cultured cells without causing the cells to come off the slide.
7. Inactivation of endogenous peroxidase is not always necessary, depending on the cell type or tissue used. The necessity of this step should be ascertained by testing positive and negative sample controls in the TUNEL assay with and without the peroxidase inactivation step.
8. The "A" (avidin DH) and "B" (biotinylated peroxidase) solutions are packaged in drop bottles with instructions to dispense drops for mixing the ABC solution. Smaller volumes of these solutions can be used in the assay, as described, by using the cap to "pull and wiggle" off the dropper piece, then using a pipetter to remove the desired aliquot.

9. Incubation with the BSA solution decreases nonspecific binding of the ABC solution to nonbiotinylated sites.
10. DAB kit reagents will darken over time and are not recommended for use after 1 yr of storage by the manufacturer. We have found that these reagents can be used longer (2+ yr) if the staining solution is filtered (0.2-μm syringe-top filter) prior to developing. The developing times may be shorter with older reagents, before background staining appears, but in general good contrast can still be achieved.
11. Developing times should be determined by the degree of background staining in negative controls (e.g., mock-infected or no treatment, NOT TdT-less or DNAse I assay controls), and the contrast between positive and negative cells in samples. When the strongest contrast is observed in samples, and negative controls do not exhibit "false-positive" background staining, development should be stopped. Sample slides and their correlating positive and negative control slides should be developed for the same length of time for appropriate comparisons.
12. DAB is a suspected carcinogen and should be disposed of according to the manufacturer's instructions.
13. Aqua PolyMount mounting medium is an aqueous solution allowing for removal of cover slips at later times for additional staining procedures. To remove cover slips, soak slides in PBS until solution softens and cover slips can be gently removed. If slides have been stored for more than several weeks, they may have to soak for up to 24 h before cover slips will loosen.
14. Cy-2 is a photostable green-fluorescing dye like fluorescein isothiocyanate (FITC), that is excited maximally with a peak at 492 nm, and fluoresces with a peak at 510 nm. Cy-2 can be effectively visualized using FITC filter sets.
15. When taking photographs or saving digital images of results, exposure times for views from sample slides and their correlating positive and negative control slides should be the same for appropriate comparisons.

Acknowledgments

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References

1. Gavrieli, Y., Sherman, Y., and Ben-Sasson, S. A. (1992) Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell. Biol.* **199,** 493–501.
2. Schwartzman, R. A. and Cidlowski, J. A. (1993) Apoptosis: the biochemistry and molecular biology of programmed cell death. *Endocrine Rev.* **14,** 133–151.
3. Majno, G. and Joris, I. (1995) Apoptosis, oncosis and necrosis. An overview of cell death. *Am. J. Pathol.* **146,** 3–15.
4. Oberhaus, S. M., Smith, R. L., Clayton, G. H., Dermody, T. S., and Tyler, K. L. (1997) Reovirus infection and tissue injury in the mouse central nervous system are associated with apoptosis. *J. Virology* **71,** 2100–2106.

5. Negoescu, A., Lorimier, P., Labat-Moleur, F., Azoti, L., Robert, C., Guillermet, C., et al. (1997) TUNEL: Improvement and evaluation of the method for in situ apoptotic cell identification. *BMB Biochemica* **2,** 12–17.
6. Prophet, E. B., Mills, B., Arrington, J. B., and Sobin, L. H. (1992) *Laboratory Methods in Histotechnology*. American Registry of Pathology, Washington, DC.
7. Virgin, H. W. IV, Bassel-Duby, R., Fields, B. N., and Tyler, K. L. (1988) Antibody protects against lethal infection with neurally spreading reovirus type 3 (Dearing). *J. Virology* **62,** 4594–4604.

II

Manipulation and Detection of Oncogenic Signals

8

Kinetworks™ Protein Kinase Multiblot Analysis

Steven Pelech, Catherine Sutter, and Hong Zhang

Abstract

The proteomics analysis of protein kinases and other cell-signaling proteins in tumor samples by traditional two-dimensional (2-D) gel electrophoresis is complicated by the low abundance of these regulatory proteins relative to metabolic enzymes and structural proteins. We present an antibody-based method called Kinetworks™ that relies on sodium dodecyl sulfate (SDS)-polyacrylamide minigel electrophoresis and multilane immunoblotters to permit the specific and quantitative detection of 45 or more protein kinases or other signal transduction proteins at once. The technique can also permit the resolution of these proteins based on differences in their phosphorylation state and other forms of covalent modification. Kinetworks™ profiling of protein kinases in solid human tumors and cell lines can reveal profound differences in their expression and phosphorylation states, which can serve for the identification of cancer diagnostic markers and therapeutic targets for drug discovery.

Key Words: Protein kinases; signaling proteins; antibodies; gel electrophoresis.

1. Introduction

More than 20 years of research has reinforced the view that cancer is rooted in mutations of key signaling proteins that are encoded by oncogenes and tumor suppressor genes. Neoplastic transformation arises when complementary tumor suppressor proteins relinquish function and oncoproteins experience a gain of function as a consequence of mutation. More than 50 known oncogenes encode protein kinases, and these represent a small subset of the more than 500 different

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protein kinases that are encoded by the human genome. They operate within complex systems of functionally interconnected signaling proteins, but the composition and architecture of these networks remains largely unknown. The pharmaceutical industry has come to appreciate protein kinases as exciting targets for disease diagnosis and drug discovery. However, large patient variation in the precise combination of molecular lesions that result in cancer requires the development of methods to track large numbers of different signaling proteins at once with limiting amounts of tissue biopsy material. This chapter describes a novel method to monitor the simultaneous regulation of 45 protein kinases or other signaling proteins in tissue biopsy and cellular extracts.

As the focus of cell-signaling research has begun to broaden into the examination of discreet regulatory pathways and their crosstalk, there is an increasing need for methods by which larger numbers of signaling proteins can be tracked reliably, quantitatively, and cost effectively. Such unbiased analyses should uncover those signaling proteins that undergo the most striking changes in expression, posttranslational regulation, or subcellular location as a consequence of perturbation of cells with diverse stimuli. Such proteins may be particularly relevant in mediation of the actions of hormones, drugs, and other stimuli within experimental model systems.

Protein phosphorylation catalyzed by protein kinases is the major form of more than 50 possible types of posttranslational modification of proteins. Such modifications cannot be monitored by nucleic acid-based approaches such as gene chips, gene arrays, or differential display of mRNA. However, phosphorylation of a protein can produce marked changes in its mobility on electrophoresis gels. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) has become the standard method in the field for separation of proteins on the basis of their size for analytical and preparative purposes. This powerful technique relies of the sieving effect of the polyacrylamide gel when proteins coated with the negatively charged detergent SDS are drawn through the gel in an electric field. Smaller-sized proteins are able to migrate through the gel faster than larger sized proteins. Proteins that differ by as little as a few hundred Daltons can be resolved by this method. Protein-staining methods permit the visualization of discreet proteins in the gel as individual bands in a bar code-like pattern. When these proteins are transferred from the SDS-PAGE gel onto a nitrocellulose or PVDF membrane, the locations of specific proteins can be identified with antibodies by an immunoblotting procedure referred to as Western blotting. Clearly, the most sensitive and specific proven reagents for the detection of proteins are antibodies. The high demand for often expensive primary antibodies underscores the importance of these tools for signal transduction research. Unfortunately, the vast majority of commercial antibodies fail to perform according to

their suppliers' claims. More than 80% of the 1300 commercial antibodies that Kinexus has independently tested have proven to be highly nonspecific and/or impotent.

Most proteomic analytical methods are based on 2-D gel electrophoresis by the standard method of Dr. Patrick O'Farrell described over 25 years ago ***(1)***. This 2-D gel technique initially involves the separation of proteins based on their intrinsic charge in a pH gradient within a tube gel. Proteins migrate through the isoelectric focusing gel in the presence of an electric field until they encounter a pH at which the protein no longer possesses a net electric charge. This pH is known as the isoelectric point of a protein, and it is a distinguishing characteristic. Following electrophoresis in the first dimension, the isoelectric focusing tube gel is applied length-wise to the top of an SDS-PAGE gel, and electrophoresis is continued into the second dimension. When the 2-D gel is stained with sensitive-dyes (e.g., based on silver reagent), the various proteins inside a cell can be visualized as resolved spots. The greater amount of a given protein within a cell sample, the larger and darker its specific spot appears. Several thousand proteins can be distinguished from one another by this technique. If the protein samples have been obtained from cells that have been incubated with radioactive ^{32}P-phosphate, then the 2-D gel can be exposed to X-ray film, and the phosphoproteins can be specifically detected by autoradiography. The more a protein is phosphorylated or prevalent, the larger and more intense the spot on the X-ray film. The silver-staining of a 2-D gel can be used to track the expression of proteins and their covalent modification by phosphorylation. When a protein is phosphorylated, its intrinsic charge is altered, and this results in a shift in the migration position of the protein in the 2-D gel. For protein spots that can be detected and unambiguously identified, the O'Farrell 2-D gel approach is a powerful way of monitoring the expression and regulation of potentially hundreds of proteins simultaneously.

Public web-based databases permit the identification of more than a thousand different proteins on 2-D gel proteomic maps. However, the positions of scarcely more than two dozen protein kinases are available. This reflects the fact that like most signal transduction proteins, protein kinases are present at very minute levels in cells, and are often undetectable by the most sensitive protein stains. Typically, transduction proteins are commonly expressed at a hundred- to a thousandfold lower levels than structural proteins and metabolic pathway enzymes. Therefore, it is often necessary to incorporate selective enrichment techniques, such as antibody-based purification as a prelude to 2-D gel electrophoresis. It is possible to visualize specific protein kinases on 2-D gels by immunoblotting techniques. However, studies in our laboratory have demonstrated that only four or five protein kinases can be detected at a time by Western blotting of 2-D

gels with mixtures of protein kinase-specific antibodies. Furthermore, we have observed that the recovery of many protein kinases from the first-dimension pH-gradient tube gel is less than 20%. That means that 80% or more of these particular protein kinases do not enter the SDS-PAGE gel, perhaps as a consequence of precipitation at their isoelectric point. This severely handicaps the use of 2-D gel electrophoresis for the analysis of low abundance signaling proteins.

A multiimmunoblot analysis strategy, as used in the Kinetwork™ analysis developed in our laboratory offers many other advantages over standard 2-D gel proteomic methods. The technique can be applied to any cell or tissue sample, including patient biopsy material. No prelabeling with radioisotopes is necessary, because protein detection is based on immunoreactivity. Phosphorylation changes can be visualized by reduced mobility of proteins in SDS-PAGE gels or by employment of phosphorylation site-specific antibodies. The Kinetwork™ analysis procedure can be carried out within 2 d from start to finish. By contrast, the 2-D gel electrophoresis approach is extremely laborious, much more difficult to render, and takes at least twice the time.

One of the reasons why application of 2-D gel electrophoresis has become the industry standard for proteomic analysis is the remarkable resolving power of this method. Proteins are visualized as spots and potentially thousands of spots can be distinguished on a 2-D gel. Most of these spots, however, are fuzzy in appearance and can be overlapping. The Kinetworks™ multiblot analysis method actually provides much tighter protein bands with two to fourfold better resolution in the SDS-PAGE size-separation dimension. Yet, it still provides resolving capability in a second dimension based on immunoreactivity as opposed to differences in the intrinsic charge of proteins. With detection based on, for example, protein kinase immunoreactivity, the background of metabolic enzymes and structural proteins is essentially eliminated. This background can be problematic even for 2-D gel maps of [^{32}P]-labeled phosphoproteins, because one-third of all the proteins inside of cells appear to be phosphorylatable.

The Kinetwork™ protein kinase multiblot analysis method is cheaper, faster, more sensitive for specific detection of protein kinases, more versatile, and more reproducible than conventional 2-D gel approaches. One of the beneficial side-effects of the Kinetwork™ multikinase analysis is that unknown proteins that can crossreact with the kinase-specific antibodies are detected. Those unidentified proteins that change in their abundance or their phosphorylation state in response to a disease condition or treatment are worthy of closer analysis. If these proteins can be shown to bind to adenosine triphosphate (ATP)-agarose or capable of autophosphorylation with radioactively labeled ATP, then there is a high probability that they are protein kinases. Moreover, with the antibody that was originally used to detect a putative kinase, it is possible to rapidly purify

the protein so that it can be sequenced by the Edman degradation method or identified by MALDI-TOF mass spectrometry of trypsin digested fragments of the protein. The availability of all of the predicted sequences of proteins encoded by the human genome makes this feasible. In this manner, novel protein kinases that display desirable characteristics (e.g., increased expression in solid tumors relative to adjacent, normal tissue) can be detected and identified. If the inappropriate activity of such protein kinases could be shown to contribute to the development of disease, then they may be valuable drug targets for the pharmaceutical industry.

2. Materials

2.1. Preparation of Lysates of Cells and Tissues

1X Lysis Buffer:

1. 20 m*M* 3-[N-morpholino]propanesulfonic acid (MOPS; pH 7.0; any other suitable buffer at this pH could be substituted);
2. 2 m*M* ethylene *bis* (oxyethylenenitrilo) tetraacetic acid (EGTA; to bind calcium);
3. 5 m*M* ethylene diamine tetraacetate disodium salt (EDTA; to bind magnesium and manganese);
4. 30 m*M* sodium fluoride (to inhibit protein-serine phosphatases);
5. 40 m*M* β-glycerophosphate (pH 7.2; to inhibit protein-serine phosphatases);
6. 10 m*M* sodium pyrophosphate (to inhibit protein-serine phosphatases);
7. 2 m*M* sodium orthovanadate (to inhibit protein-tyrosine phosphatases);
8. 1 m*M* phenylmethylsulfonylfluoride (to inhibit proteases, can be omitted);
9. 3 m*M* benzamidine (to inhibit proteases, can be omitted);
10. 5 μ*M* pepstatin A (to inhibit proteases, can be omitted);
11. 10 μ*M* leupeptin (to inhibit proteases, can be omitted); and
12. 0.5% Nonident P-40 (NP40; can be substituted with 0.5% Triton X-100; do not add if intention is to first prepare a cytosolic fraction).

The final pH of the homogenizing buffer should be adjusted to 7.0.

2.2. Gel Electrophoresis

1. Stacking Gel: 3% Tris-HCl hydroxylmethyl aminomethane hydrochloride (Tris-HCl, pH 6.8; w/v), 4.0% acrylamide (w/v), 0.1% *bis*-acrylamide (w/v), 0.1% SDS (w/v), 0.1% TEMED (v/v, added as a catalyst for polymerization), 0.1% ammonium persulphate (w/v, added as a catalyst for polymerization).
2. Separating Gel: 7.25% Tris-HCl Base (w/v), 2.38% Tris-HCl (w/v), 12.5% acrylamide (w/v), 0.083% *bis*-acrylamide (w/v), 0.1% SDS (w/v), 0.4% TEMED (v/v, added as a catalyst for polymerization), 0.1% ammonium persulphate (w/v, added as a catalyst for polymerization).

3. 5X SDS-PAGE Sample Buffer: 150 m*M* Tris-HCl (pH 6.8), 62.5% glycerol (v/v), 5% SDS (w/v), 0.1% bromophenol blue (w/v), 5% β-mercaptoethanol (v/v).
4. 10X Running Buffer: 14.42% glycine (w/v), 3% Tris-HCl Base (w/v), 1% SDS (w/v).
5. 10X Transfer Buffer: 2.4% Tris-HCl Base (w/v), 9% glycine (w/v).
6. 10X Tris-HCl-Buffered Saline (TBS): 2.42% Tris-HCl Base (w/v), 14.61% sodium chloride (w/v).
7. 1X Tween Tris-HCl-Buffered Saline (TTBS): 0.242% Tris Base (w/v), 1.46% sodium chloride (w/v), 0.05% Tween-20 (v/v).
8. 1X Blocking Solution: 2.5% skim milk powder (w/v), 1.5% bovine serum albumin (BSA) (w/v), 0.242% Tris-HCl Base (w/v), 1.46% sodium chloride (w/v), 0.05% Tween-20 (v/v).
9. Incubation of multiblot with primary antibodies: Commercially sourced primary rabbit and goat polyclonal antibodies and mouse monoclonal antibodies that have been well tested for specificity and potency for target protein kinases. Each antibody is used at approx 1:1000 or 1 µL per mL of dilutent.
10. Incubation of multiblot with secondary antibodies: Commercially sourced secondary donkey antirabbit antibody: 1:5000 or 0.2 µL per mL of dilutent; sheep antimouse antibody—1:10000 or 0.1 µL per mL of dilutent; bovine antigoat antibody—1:10000 or 0.1 µL per mL of dilutent. The secondary antibodies are fused with horseradish peroxidase to facilitate detection of the locations to which the primary antibodies bound.
11. Enhanced chemiluminescence (ECL) Plus kit from Amersham-Pharmacia.

3. Methods

The methodology behind the Kinetwork™ multiimmunoblot analysis is relatively simple in principle. However, it requires access to a wide range of specific antibodies and extensive experimentation to optimize the separate mixtures of antibodies for the analysis. Here, we describe the protocols for a specific multiblot for detection of a wide range of protein-serine kinases. Such procedures can be adapted for analyses of many other classes of signaling proteins.

3.1. Preparation of Lysates of Cells and Tissues

1. For examination of total levels of protein kinases, lysis and homogenization should be performed at 4ºC in the presence of a detergent such as 0.5% NP40 (*see* **Note 1**). Detergents should be omitted from the initial homogenization buffer if the subcellular distribution of kinases is to be examined. In this instance, the detergent should be added to the microsomal pellet for solubilization of membrane-bound proteins after removal of the cytosolic extract.
2. For cells, use about 0.5 mL of lysis buffer per sample. For example, ten million cells in a pellet in a 50-mL Falcon tube following refrigerated centrifugation at 1000*g* for 5 min would have 0.5 mL of lysis buffer added. This should be sonicated twice for 15 s each time to rupture the cells. Alternatively, homogenization

could be performed with 10 full strokes with a glass Dounce. The homogenate should ideally be subjected to ultracentrifuge for at least 30 min at 100,000*g* or more. This can be achieved, for example, with a Beckman Table Top TL-100 ultracentrifuge or Beckman Airfuge. The resulting supernatant fraction should be removed and immediately assayed for its protein concentration.

3. For tissues, use 4 mL of lysis buffer per 1 g wet weight of the chopped tissue. Homogenization should be performed with 10 full strokes of a motor driven Van Potter homogenizer (or 15 s with a Brinkman Polytron Homogenizer or with a French Press as alternatives). The homogenate should ideally be subjected to ultracentrifugation for at least 30 min at 100,000*g* or more. The resulting supernatant fraction should be removed and immediately assayed for its protein concentration.
4. If a particulate-solubilized fraction is to be analyzed, a microsomal pellet should be obtained following the initial homogenization and ultracentrifugation in the absence of detergent and subsequent removal of the cytosolic fraction. The microsomal pellet should then be resuspended in the homogenization buffer containing 0.5% NP40 using a sonicator, glass dounce, or Polytron homogenizer. The detergent-treated microsomes should be subjected to ultracentrifugation for 30 min at 100,000*g* or more. The resulting detergent-solubilized microsomal fraction should be removed and immediately assayed for its protein concentration.
5. The protein concentrations of the cell/tissue extract samples should be determined using a commercial Bradford Assay (available from Bio-Rad) or using the standard protocol of Bradford ***(2)***. BSA could be used as the protein standard.

3.2. Gel Electrophoresis and Immunoblotting

1. We suggest the use of the Bio-Rad Mini-PROTEAN 3 gel casting unit and electrophoresis system and following the manufacturer's recommendations (*see* **Note 2**). To optimize the detection of protein band shifts that reveal phosphorylation, the 0.75-mm-thick SDS-PAGE minigel should be precast with a higher ratio of acrylamide to *bis*-acrylamide than normal (i.e., final ratio of 150:1, w/w) (*see* **Note 3**). The stacking gel should have 7.5% acrylamide and the separating gel should contain 12.5% acrylamide.
2. The cell/tissue sample should be diluted with 5X SDS-PAGE Sample Buffer (*see* **Note 4**). The final protein concentration of the cell/tissue samples should be 1 mg/mL in 1X SDS-PAGE sample buffer as specified by Laemmli ***(3)***. The cell/tissue sam-ple should be boiled for 4 min at 100ºC in 1X SDS-PAGE sample buffer.
3. Boiled cell/tissue lysate extract containing about 250 µg of protein in 1X SDS-PAGE sample buffer should be loaded on to the stacking gel of the mini SDS-PAGE gel in a single lane that spans the width of the gel. The closed mini-gel apparatus should be placed in ice water prior to the commencement of electrophoresis (*see* **Note 5**). An electric current (25 mA per gel) should be applied to the slab gel for approx 1.5 h until proteins with a molecular mass less than 27 KDa are eluted from the bottom of the gel; known protein kinases do not exist with molecular masses

less than this (*see* **Note 6**). The proteins remaining on the slab gel are then electrotransferred (250 mA per tank for 30 min) on to a thin membrane (nitrocellulose or PVDF) that traps the proteins (*see* **Note 7**).

4. Rehydrate the immunoblot membrane in 1X TBS and place the blot in TBS, on the shaker for a few minutes to remove any Ponceau stain that may have been added to visualize proteins (*see* **Note 8**). Approximately 10 mL of 1X blocking solution should be made for the blot to be probed. The blocking solution should contain 2.5% skim milk powder and 1.5% BSA in 1X TTBS. Place the blot with the blocking solution into a shaking tray (*see* **Note 9**). Following agitation for 30 min, pour off the blocking solution and rinse twice with 1X TTBS. Add fresh 1X TTBS, shake for an additional 15 min, and perform two additional 5-min washes with fresh changes of 1X TTBS.
5. Using a 20-lane multiblotter apparatus from Bio-Rad, the immunoblot membrane can be probed with different mixtures of up to three primary antibodies per mix that react with a distinct subset of protein kinases of distinct molecular masses (*see* **Note 10**). Using forceps, place the blot, protein side down, onto the lane half of the miniblotter. Align the lanes with left and right markings on the membrane. Place a cushion behind the blot, making sure the top and bottom of each lane is covered with the cushion. Using the miniblotter screws, secure both halves of the miniblotter together. Load 600 μL of the appropriate antibody mix into each lane of the miniblotter. When finished, cover the miniblotter with plastic wrap, and place into the 4ºC cabinet overnight. When transporting the miniblotter to a refrigerator, be sure to keep it flat, so that the antibodies in separate lanes do not mix.
6. Following incubation of the membrane with the different mixtures of primary antibodies, the immunoblot membrane is incubated with a mix of secondary antibodies that react with the F_C portion of the primary antibodies (*see* **Note 11**). After overnight incubation, remove the miniblotters from the 4ºC cabinet, and remove the plastic wrap from the miniblotters. Pour off the primary antibodies into the sink. Make sure to pour only in one direction, to avoid contaminating the lanes of the miniblotter. Remove the miniblotter screws, and separate the two halves of the miniblotter. Remove the immunoblot from the blotter and place into 1X TTBS in a shaking tray. Rinse the blots twice with 1X TTBS then add fresh 1X TTBS and wash for 15 min followed by two additional 5-min washes with fresh 1X TTBS. The appropriate secondary antibodies are then incubated together with the blot for 30 min with gentle shaking. After 30 min, pour off the secondary antibody solution, rinse twice with 1X TTBS, and incubate with fresh 1X TTBS for 15 min with shaking. Prior to ECL detection, the blot should be subjected to four more 5-min washes with fresh 1X TTBS.
7. The immunoblot membrane should be subjected to enhanced chemiluminescence (ECL) and the immunoreactive protein bands can be detected with a Bio-Rad FluorS Max Multi-imager (*see* **Note 12**). In this manner, the original positions of resolved protein kinases are visualized as light bands on a dark background. Quantitation of the intensity of signal for each immunoreactive band can be provided with Quantity One Software from Bio-Rad. Turn down the lights, and prepare the ECL reagent

in a 50-mL Falcon tube covered in foil according to the manufacturer's information. ECL reagent should be made fresh for every immunoblot. Using forceps, remove the blot from its shaking tray and blot excess liquid with a Kimwipe tissue. Place the blot on plastic transparency sheet. Slowly pipet 2 mL of mixed ECL solution and slowly disburse over the membrane. Cover and set timer for 5 min. When the last minute of the incubation begins, begin rolling the plastic transparency sheet such that the ECL solution continually covers the blot. Continue this until the timer finishes. Take the blot off of the blank transparency, and blot off the excess ECL solution using Kimwipe tissues. Place the blot onto the scanning sheet and place in a multiimager for scanning for chemiluminescence.

8. The resulting immunoblot should be analyzed for the intensity of the signal produced for each immunoreactive band and interpreted based on knowledge of the molecular masses of the target proteins and the composition of primary antibodies used in each lane (*see* **Notes 13** and **14**).

4. Notes

1. We recommend the use of 0.5% NP40 or 0.5% Triton X-100, but comparable detergents are acceptable. Important things to consider are that the cells or tissues should be processed quickly at 1–4ºC. Homogenization should not be performed in too large a volume. The detergent-soluble fraction should be obtained as quickly as possible after the cells or tissues are homogenized. The highest centrifugal forces available should be used to generate the cytosolic and detergent-solubilized fractions. The supernatants should be frozen as quickly as possible if a protein assay and Kinetworks™ multiblot analysis cannot be performed immediately.
2. The use of minigels for this Kinetworks™ multiblot analysis reduces the amount of cell/tissue lysate protein required as well as the amount of immunoblotting reagents. This provides tremendous cost savings. However, this methodology can also be performed with standard 20 × 20 cm SDS-PAGE gels.
3. Acrylamide is a neurotoxin and may cause allergic skin reaction and be harmful if absorbed through the skin or swallowed. Always wear gloves and eye protection when handling acrylamide powder and solutions.
4. Although we recommended a final protein concentration of the cell/tissue sample as 1 mg/mL in 1X SDS-PAGE sample buffer, the minimum useable protein concentration is about 0.5 mg/mL. Lower concentrations of protein create loading problems for 250 µg of protein required for the Kinetworks™ multiblot analysis.
5. SDS-PAGE is performed in ice water to prevent the formation of band distortions that are commonly referred to as "smiles."
6. The extended period of electrophoresis and the use of a higher acrylamide to *bis*-acrylamide ratio maximizes the opportunity to observe band retardation of 0.5 to 5 kDa that can arise from phosphorylation of proteins. More than 75% of the protein kinases that we have tracked display band shifts due to phosphorylation provided that cells receive the appropriate stimulation prior to harvesting.
7. To ensure proper transfer of resolved proteins from the SDS-PAGE gel onto the immunoblot membrane, verification by Ponceau staining is highly recommended.

Jurkat Tcells
Hek 293 kidney
U87M6 glioma
A549 lung
Legend
1 - RafB
2 - Erk3
3 - PKBα (Akt1)
4 - pErk1
5 - Erk1
6 - Erk2
7 - Cdk1
8 - PDK1
9 - pErk2
10 - Erk2
11 - Cdk2
12 - PKCμ
13 - PKCα
14 - Cdk4
15 - PKCβ1
16 - p38α MAPK
17 - Cdk5
18 - PKCγ
19 - Cdk6
20 - PKCε
21 - Cdk7
22 - Cdk9
23 - PKCδ
24 - PKCλ
25 - JNK (SAPK)
26 - JNK (SAPK)

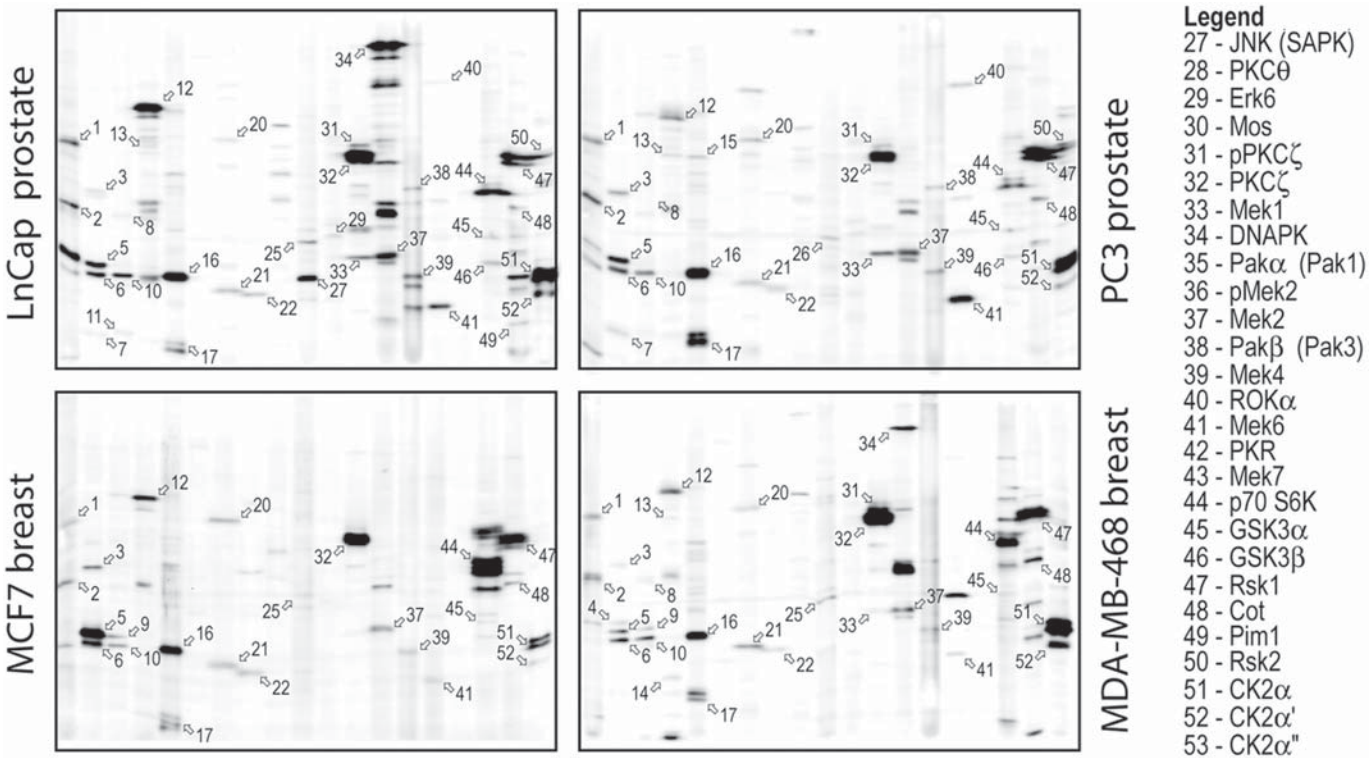

Fig. 1. Kinetworks™ Protein Kinase Screen (KPKS 1.0A) of human tumor cell lines. Established lines of tumor cells were grown in 5% fetal calf serum and Dulbecco's modified Eagle medium and harvested while the cultures were 60–80% confluent. The cells were sonicated, and 0.5% NP40-solubilized extracts were prepared and subjected to KPKS 1.0A analysis. Scans of the ECL signals detected with a Bio-Rad Fluro-S Max Multiimager are shown.

8. When rehydrating a dried immunoblot membrane in 1X TBS, take special care to avoid creation of air pockets that may interfere with rehydration.
9. Note that any blocking solutions for use with phospho-site-specific antibodies should avoid the skim milk powder. The use of skim milk with antibodies that recognize phosphorylation sites creates high backgrounds in the resulting immunoblots.
10. It is important that none of the antibodies in a given mixture crossreact with different proteins that overlap in size, because this would compromise on the interpretation of the experimental findings. Each antibody mixture has to be carefully blended to avoid such overlaps. The use of more than three primary antibodies per mix increases the prospect of crossreactive bands that comigrate with target proteins and generate false positives. Furthermore, every mixture must be adjusted for the concentration of each antibody so that there is optimal detection of the individual target kinases in diverse cell and tissues samples.
11. It is important to pretest the quality of the secondary antibodies that are used for the Kinetworks™ protein kinase multiblot analysis. Many commercial secondary antibodies can generate high backgrounds that can compromise the detection of low abundance proteins.
12. There are several scanners that are commercially available for detection of chemiluminescence. With the Bio-Rad FluorS Max Multiimager and the accompanying Quantity One software from Bio-Rad, we have been able to achieve quantitative detection of immunoreactive proteins over a 1000-fold range.
13. In many cases, quantitation of the amounts of a given protein kinase in the upper, phosphorylated form, and the lower, dephosphorylated form can provide an accurate measurement of how much of the kinase is in the inactive and active states.
14. **Figure 1** demonstrates the ability of the Kinetworks™ method to reveal profound differences in the levels of expression and phosphorylation of up to 45 different protein kinases in eight distinct established human tumor cell lines. It is evident that there can be striking kinase expression pattern differences even between cell lines from tumors of the same tissue type of origin such as breast or prostate. Certain protein kinases such as the MAP kinases Erk1, Erk2, and p38, cyclin-dependent kinase 5, protein kinase C-ζ, p70 S6 kinase, Rsk1, and casein kinase 2 were commonly and very highly expressed in all of the tumor cell lines. However, others such as DNA-dependent protein kinase, protein kinase C-β and μ, cyclin-dependent kinases 6 and 9, p21-activated kinases α and β, Mek6, Cot, and Pim1 showed strong differences between the same cell lines. We have also noted increased levels of many of these kinases (e.g., p21-activated kinase-α, Mek6, p38 MAP kinase, casein kinase 2, Cot, and Pim1) in biopsy samples from human lung, breast, colon, and liver tumors relative to patient-matched control tissues (unpublished data). In many of these cases, the kinases may be up regulated in expression in the tumors as compensatory responses in a futile attempt to re-establish growth control. Therefore, it will be extremely important to use antisense expression or transfection with dominant-negative forms of these protein kinases in tumor cells to validate them as legitimate drug targets for discovery of cancer therapeutic leads.

References

1. O'Farrell, P. H. (1975) High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250,** 4007–4021.
2. Bradford, M. M. (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72,** 248–254.
3. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–684.

9

Protein Tyrosine Kinase and Phosphatase Expression Profiling in Human Cancers

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Abstract

Alterations of protein tyrosine kinase and tyrosine phosphatase are often associated with uncontrolled cell growth and cellular transformation. Because of the large number of tyrosine kinase/phosphatase genes in such gene family, it is essential to use an efficient and simple approach to obtain comprehensive protein tyrosine kinase and protein tyrosine phosphatase expression profiles. Knowledge of such an overall expression pattern of tyrosine kinases/phosphatases in a given cancer cell represents the first step in understanding key components involved in the sequential events of tumor progression. In this article, we described a novel approach by using degenerate PCR primers according to the consensus catalytic motifs in order to amplify protein tyrosine kinase/phosphatase molecules from cancer cells by reverse-transcription polymerase-chain-reaction. An improved profiling approach (RAGE) was also described by utilizing restriction enzyme digestion and electrophoresis for quick and efficient kinase/phosphatase profiling.

Key Words: Tyrosine kinase; tyrosine phosphatase; gene expression profile; cancer cells; PCR.

1. Introduction

Protein phosphorylation is mediated by groups of protein kinases and phosphatases that play a critical function in signal transduction, cell growth, differentiation and oncogenesis ***(1)***. Protein kinases are large families of enzymes and are involved in transferring a phosphate molecule from a phosphate donor, usually ATP, onto an acceptor amino acid in substrate proteins. They can therefore

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be classified into several subgroups according to the acceptor amino acid residues, including tyrosine kinases *(2)*. Protein tyrosine kinase (PTK) is the most significant protein kinase subgroup, the abundance of PTKs accounts for less than 10% of all cellular kinases, nearly all of them are involved in cell growth signaling. Many of the receptor type PTKs belong to growth factor receptors, and a number of the nonreceptor type PTKs are directly associated with growth factor receptors. Therefore, it has been suggested that PTKs participate in signal transduction pathways as key regulators and thus play critical roles in the regulation of cell growth and oncogenesis *(3)*. Many of PTKs have been shown to be oncogenic in vivo or to be transforming in vitro, and numerous examples of PTK abnormalities in human tumors were also reported on their overexpression, translocation and mutations *(4–6)*. Therefore, knowledge about the PTKs expressed in human cancer cells represents a critical step in understanding the oncogenic mechanisms of human cancer *(7)*.

On the other hand, protein-tyrosine phosphatases (PTPs) attenuate growth signals generated by PTKs through catalyzing the tyrosine dephosphorylation step on their substrate proteins *(8)*. We should bear in mind that phosphorylation on the tyrosine residues is carefully balanced in cells by the actions of PTKs and PTPs, and this reversible and dynamic biochemical mechanism act as the molecular switches of signal transduction pathways *(1,9)*. Although the understanding of PTPs has somewhat lagged behind that of PTKs, studies on the PTPs have progressed rapidly in recent years. Many PTPs are implicated in the oncogenic process in human cancer cells as tumor suppressor genes, such as PTEN tyrosine phosphatase *(10)*. Furthermore, PTPs also play fundamental roles in cell-cycle regulation and activation *(8)*. In addition, it is well documented that cross-talk is involved in the kinase–kinase and kinase–substrate interactions *(11)*. It is essential to identify the PTKs and PTPs expressed in cells to give a representative picture. Therefore, an efficient approach needs to be implemented to examine the complexity of PTK/PTP expression in various human cell types *(7)*. It is unlikely to examine the expression of PTK and PTP genes individually, because the PTK and PTP families are among the largest gene families in the human genome *(3,12)*. In order to learn about the complete regulatory mechanisms in the signal transduction pathways, such a general and comprehensive PTK expression profile in a particular cell type will be beneficial. The information obtained will help to identify PTK and PTP genes involved in the development of cancers.

It is critical to identify as many PTKs and PTPs expressed in cells as possible. High-throughput approaches including EST based expression profile *(13)*, SAGE-Serial Analysis of Gene Expression *(14)* and cDNA microarray *(15)* have been used to study global gene expression patterns, but these approaches are not suitable economically for establishing specific PTK and PTP expression

profiles in cancer cells. Since we are interested only in the tyrosine kinase and phosphatase genes, it is more efficient and inexpensive to identify expressed PTKs and PTPs in a single polymerase chain reaction (PCR) reaction. In collaboration with Dr. Hsing-Jien Kung at University of California-Davis, we have adapted his reverse transcription (RT)-PCR-based tyrosine kinase profiling approach for human gastric cancers, which uses degenerate PCR primers that permit the amplification of all PTKs and PTPs ***(16)***.

As completion of human genome project, it is estimated that there are at least several hundred protein kinase genes encoded by the human genome and about one hundred of them are tyrosine kinases ***(12,17)***. The number of tyrosine phosphatase genes is slightly lower at around 50 PTPs in the human genome ***(17)***. With half of PTKs expressed in a given cell at a given time, it is reasonable to assume that 30–50 PTKs are expressed and can be detected. This number is large enough to give a fingerprint of a cancer cell, but small enough to be identified in a simple assay. Robinson et al. ***(16)*** designed a set of primers based on motifs in subdomain VII and IX of the catalytic domain of tyrosine kinases and optimized the conditions, such that the reaction is of high fidelity and broad specificity. Similar PCR approaches have been used before for isolating novel PTK genes by several groups, but not optimized for expression profiling ***(18, 19)***. Subdomains VI (xHRDxKxx) and IX (DVWxxG) have been frequently used and Wilks et al. identified six different PTK genes among 200 clones analyzed ***(20)***. Mossie et al. identified 26 PTK genes in 250 PCR clones analyzed with HRDLAA and D(V/M) WS(F/Y)G primers ***(21)***. Takahashi et al. used the HDLAAR and DVWSFGV primers and identified 24 PTK genes in 37 clones ***(22)***. In a detailed study ***(23)***, Schultz et al. used 11 degenerate primers covering subdomains V to IX to isolate PTK/protein serine kinase (PSK) genes. A total of 41 PTK/PSK genes were identified from 316 clones. For the PTP profile, KCXXYWP and HCSXGXG in the PTP domain were used ***(24)***. The success rate of identifying PTK/PSK related genes ranged from 33% to 90% in total. In summary, the efficiency of degenerate PCR amplification is influenced little by the primer length, but is greatly affected by primer degeneracy, with lower degeneracy being most favorable.

In order to increase the varieties of PTK genes identified without sacrificing the PCR amplification efficacy, we used three forward PCR primers corresponding to subdomain VII and one reverse primer for subdomain IX ***(25)***. By this approach, the incubation temperature of our PCR reactions could be raised from 37ºC to 44ºC during the first five cycles and 56ºC during the following 25 cycles. This certainly increased the specificity in identifying PTK/PTP genes ***(26–28)***. In our study, more than 95% of the sequences identified were kinase-related genes. In addition, the relatively homogeneous size distribution between subdomains VII and IX made purification of the PTK specific amplicon possible.

This enhances the specificity of our PTK/PTP profiling approach by eluting the amplicon fragment from gels before further analysis. Following the purification of amplicon, PTK/PTP genes were identified by cloning and sequencing procedures. A variation of this procedure utilizing restriction digestion and gel display analysis of the amplicon, instead of cloning and sequencing, was recently developed *(5,7)*. This method combines the special features of the RT-PCR/degenerate-primer approach described above and the differential display approach which separates individual genes on a sequencing gel *(5)*. Briefly, by radio-labeling the 5'-end of forward primers, subjecting the resulting amplicon to restriction digestions, and resolving the 5' end fragments in a sequencing gel, individual tyrosine kinases can be identified by the characteristic size of their 5' end fragments. Because each molecule is labeled only at the 5'end, the relative intensity of the displayed fragments reflects the approximate molar ratio of the tyrosine kinase transcript copies. This approach, which does away with cloning and sequencing, should greatly improve the quantitative aspects of the procedure. The intensity of individual bands visible in the phosphoimager-processed files can be used to quantify the expression level of the original transcripts *(5)*. Thus, using a single RT-PCR reaction and using a single gel, a tyrosine kinase profile can be obtained. The development of these tyrosine kinase profiles provides a good framework to study the functions of these kinases in carcinogenesis, as well as to identify potential predictive markers and intervention targets.

As examples, we have applied this expression profiling approach to establish PTK/PTP expression profiles in matching pairs of human gastric cancers *(27, 28)*. The data reveals the expression of more than 30 different tyrosine kinases and a few dual kinases in human gastric cancer tissues. These studies identified a number of differentially expressed tyrosine kinases that were previously unrecognized in human gastric cancer cells. Further immunohistochemical studies, however, showed that *tie-1*, a receptor tyrosine kinase thought to have a restricted expression in endothelial and hematopoietic cells, is actually expressed in gastric adenocarcinoma cells as well. Moreover, the aberrant expression of *tie-1* in gastric cancer cells was shown to be associated with reduced survival of the patients and serve as an independent predictor for survival *(27)*. Likewise, the presence of *mkk4* kinase by immunohistochemistry was shown also to be an independent prognostic factor for human gastric cancers *(28)*.

2. Materials

2.1. Total RNA Isolated From Cell Lines or Tissues (25 µg)

Total RNA can be isolated from cells with the any RNA extraction reagent, stored in 100% formamide at –80ºC, precipitated with ethanol and redissolved in DEPC treated water right before use. Immediately before PTK/PTP profiling,

integrity and quality of purified total RNA should be assessed by gel electrophoresis or by PCR with positive-control actin gene primers.

2.2. RT of First Strand cDNA

1. Oligo dT25 anchor primer, 0.5 µg/µL.
2. Avian Myeloblastosis Virus Reverse Transcriptase (AMV RT), 5 U/µL.
3. AMV RT buffer 5X: 250 m*M* Tris-HCl, 40 m*M* $MgCl_2$, 5 m*M* dithiothreitol, 150 m*M* KCl, pH 8.5.
4. dNTP nucleotide mixture: 10 m*M* each dATP, dGTP, dTTP, and dCTP.
5. RNase inhibitor, 40 U/µL.
6. 0.5 *M* ethylene diamine tetraacetic acid (EDTA), pH 8.0.
7. CHROMA spin-200 gel filtration column (CLONTECH).

2.3. PTK/PTP Amplicon Amplification by Polymerase Chain Reaction

1. Recombinant *Taq* DNA polymerase, 5 U/µL.
2. 10X PCR reaction buffer: 200 m*M* Tris-HCl and 500 m*M* KCl, pH 8.3.
3. 50 m*M* $MgCl_2$.
4. dNTP nucleotide mixture, 10 m*M* each dATP, dGTP, dTTP, and dCTP.
5. PTK profile degenerate primers ***(29)***.
   Sense primers: 5'-CAGGTCACCAARRTIDCNGAYTTYGG-3'
   5'-CCAGGTCACCAARRTTDCNGAYTTYGG-3'
   Antisense primer: 5'-CACAGGTTACCRHAIGMCCAIACRTC-3'
6. PTP profile degenerate primers ***(24)***.
   Sense primers: 5'-CAGTGGATCCAARTGYGMIMRRTAYTGGCC-3'
   Antisense primer: 5'-CTAGGAATTCCIRYRCCIGCRCTRCARTG-3'
7. The mixed bases were defined as follows: N = A + C + T + G; D = A + T + G; H = A + T + C; V = A + G + C; R = A + G; Y = C + T; M = A + C; and I =deoxyinosine.

2.4. End-Labeling Sense Primers by T4 Polynucleotide Kinase

1. 10X T4 polynucleotide kinase buffer: 700 m*M* Tris-HCl, 100 m*M* $MgCl_2$, 50 m*M* dithiothreitol, pH 7.6.
2. PTK and PTP sense primers, 10 µ*M* each.
3. T4 polynucleotide kinase, 10 U/µL.
4. γ-^{33}P ATP , 10 µCi/µL.

2.5. PTK/PTP Amplicon Purification, Cloning, and Sequencing (Unlabeled Sense Primer)

1. 3% NuSieve 3:1 agarose gel (FMC).
2. 1X TAE buffer: 40 m*M* Tris/acetate, 1 m*M* EDTA, pH 8.0.
3. 6X gel-loading dye: 0.25% bromophenol blue, 0.25% xylene cyanol, 15% Ficoll type 400 in H_2O.
4. DNA fragment extraction kit.

5. TE buffer: 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0.
6. T-vector plasmids.
7. 10X T4 DNA ligase buffer: 660 m*M* Tris-HCl, 50 m*M* $MgCl_2$, 10 m*M* dithioerythritol, 10 m*M* adenosine triphosphate (ATP), pH 7.5.
8. T4 DNA ligase, 1 U/μL.
9. Competent bacterial cells, high efficiency.
10. Plasmid DNA extraction kit.
11. GenBank, BLAST: http://www.ncbi.nlm.nih.gov/.

2.6. PTK/PTP Amplicon Purification, Restriction Enzyme Digestion and Gel Display (Labeled Sense Primer)

1. 3% NuSieve 3:1 agarose gel (FMC).
2. 1X TAE buffer: 40 m*M* Tris/Acetate, 1 m*M* EDTA, pH 8.0.
3. 6X gel-loading dye: 0.25% bromophenol blue, 0.25% xylene cyanol, 15% Ficoll type 400 in H_2O.
4. DNA fragment extraction kit.
5. TE buffer, 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0.
6. 10X restriction enzyme buffer.
7. Restriction enzyme panel: *AluI, CfoI, DdeI, HaeIII, HinfI, MnlI, MspI, Sau3aI, Sau96I, ScrfI, Tru9I,* and *RsaI*.
8. Formamide loading dye: 100% formamide, 0.025% bromophenol blue, 0.025% xylene cyanol.
9. 7% polyacrylamide/urea sequencing gel.
10. ^{35}S-dATP labeled pre-determined DNA sequence ladder as size maker.

3. Methods

3.1. RT of First Strand cDNA

1. 25 μg total RNA is precipitated, centrifuged and dissolved in 10 μL DEPC H_2O.
2. 1 μL oligo dT primer and 5 μL DEPC H_2O are added to the RNA tube, and incubated at 65ºC in a water bath for 10 min. Transfer the reaction tube onto ice following the 65ºC RNA denaturation step.
3. Add 5 μL 5X AMV RT buffer, 2.5 μL dNTP nucleotide mixture, 0.5 μL RNase inhibitor, and 1 μL AMV RT enzyme to bring up to a total 25 μL reaction volume. Mix well and spin briefly in an Eppendorf centrifuge.
4. Incubate at 42ºC in a water bath for 30 minutes. Stop the reaction by adding 1 μL 0.5 *M* EDTA solution.
5. Bring the total volume to 65 μL by adding DEPC H_2O, and remove RT enzymes by phenol/chloroform extraction once and chloroform extraction once. Transfer the aqueous phase to a fresh Eppendorf tube.
6. Spin the CHROMA spin-200 column to remove buffer, and load the first strand RT reaction mixture onto the CHROMA spin-200 column. Centrifuge the column again to separate the first strand cDNA products and unincorporated dNTP mixture

and primers. Collect the effluent in a fresh Eppendorf tube, and store the first strand RT cDNA template at –80ºC if necessary.

3.2. PTK/PTP Amplicon Amplification by PCR (see Note 1)

1. Add the following components to a sterile 0.2 mL thin-wall PCR tube sitting on ice:
   a. 10X PCR reaction buffer 5.0 µL
   b. 50 m*M* $MgCl_2$ 0.8 µL
   c. 10 m*M* dNTP mixture 1.0 µL
   d. Labeled or unlabeled 5' primer 5.0 µL
   e. Unlabeled 3' primer 5.0 µL
   f. First strand cDNA template 5.0 µL
   g. Add DEPC H_2O to bring the total reaction volume to 49 µL.
2. Mix well and overlay with 50 µL mineral oil. Centrifuge briefly to collect the content to the bottom.
3. Incubate the reaction tube in a PCR thermal cycler at 96ºC for 3 min to completely denature the template.
4. Set the thermal cycler at 68ºC following the denaturation step.
5. Add 1 µL *Taq* DNA polymerase to the bottom of reaction mixture, and mix gently.
6. Perform four cycles of PCR amplification as follows:
   a. Denature 93ºC for 45 s
   b. Anneal 44ºC for 90 s
   c. Extend 72ºC for 12 s
7. Perform an additional 26 cycles of PCR amplification as follows:
   a. Denature 93ºC for 45 s
   b. Anneal 56ºC for 90 s
   c. Extend 72ºC for 15 s
8. Incubate for an additional 10 min at 72ºC and maintain the reaction at 4ºC until subsequent purification.

3.3. End-Labeling Sense Primers by T4 Polynucleotide Kinase

1. Mix 2 µL sense primer, 1 µL 10X T4 polynucleotide kinase buffer, 5 µL γ-^{33}P ATP, 0.5 µL T4 polynucleotide kinase, and 1.5 µL H_2O into a sterilized Eppendorf tube.
2. Incubate the reaction mixture at 37ºC in a water bath for 30 min, and then incubate at 65ºC in a water bath for another 5 min to inactivate the T4 polynucleotide kinase enzyme activity.
3. The labeled sense primers were used as 10X stock solution for the PCR experiments.

3.4. PTK/PTP Amplicon Purification, Cloning and Sequencing (Unlabeled Sense Primer)

1. Prepare 3% NuSieve 3:1 agarose gel in 1X TAE buffer.
2. Load the amplified PCR products into agarose gel by mixing with loading dye.
3. After running the gel, stain with 1 µg/mL ethidium bromide, and excise the 170 bp amplicon DNA fragments from gel.

4. Purify DNA fragments from gel following the instructions of DNA fragment extraction kits.
5. Repeat the extraction steps to remove possible containments. Amplified PTK/PTP amplicon fragments are then precipitated with ethanol, centrifuged, and dissolved in TE buffer.
6. Mix 1 µL PTK/PTP amplicon DNA fragments with 1 µL 10X T4 DNA ligase buffer, 2 µL T-vector plasmid, 1 µL T4 DNA ligase and 6 µL H_2O. Incubate the ligation mixture overnight at 15ºC.
7. Perform transformation procedure with suitable competent *Escherichia coli* (*E. coli*) cells, select antibiotic resistant bacterial colonies and isolate plasmid DNA from randomly selected colonies.
8. PTK/PTP insert sequences of each plasmid are determined by DNA autosequencer. The sequences obtained can be compared with GenBank database sequences from National Center for Biotechnology Information using the BLAST program to identify each PTK/PTP gene expressed.
9. PTK/PTP expression profiles can be established by the number of different kinase/phosphatase clones identified.

3.5. PTK/PTP Amplicon Purification, Restriction Enzyme Digestion and Gel Display (Labeled Sense Primer, see Note 2)

1. Prepare 3% NuSieve 3:1 agarose gel in 1X TAE buffer.
2. Load the amplified PCR products into agarose gel by mixing with loading dye.
3. After running the gel, stain with 1 µg/mL ethidium bromide, and excise the 170 bp amplicon DNA fragments from gel.
4. Purify DNA fragments from gel following the instructions of DNA fragment extraction kits.
5. Repeat the extraction steps to remove possible containment. Amplified PTK/PTP amplicon fragments are then precipitated with ethanol, centrifuged and dissolved in TE buffer.
6. Determine the radioactivity of recovered PTK/PTP DNA fragments, and adjust the final volume to 10^4 cpm/µL.
7. Equal amounts (at least 40,000 cpm) of the eluted PTK/PTP amplicon are then digested 60 min with respective restriction enzymes listed in the panel (*see* **Subheading 2.6., item 7.**) under optimal restriction enzyme conditions.
8. 6.6 µL formamide loading dye is added to the digested products and incubated at 65ºC for 7 min before loading onto gel.
9. 4 µL of final product is applied to each lane in 0.7% denaturing sequencing polyacrylamide gel. A sequencing reaction product (^{35}S-label, and single-track sequencing only) with a predetermined sequence template is used as a standard for fragment size throughout the profile analysis. Any known sequence will be useful, because we use it as a size standard. (*see* **Notes 3–5**.)
10. Following electrophoresis, the gel is dried and exposed to a X-ray film or processed by a phosphoimager.

11. As shown in **Fig. 1**, the amplified PTK products were digested with Mwo I restriction enzyme and separated on a sequencing gel. Samples from multiple tissues could be displayed on a single sequencing gel; thus, we could effectively screen all known human PTKs/PTPs in a short period of time.
12. From the preestablished bioinformatic database of human PTKs/PTPs digested with different restriction enzymes, individual tyrosine kinases were identified based on their respective characteristic restriction fragment sizes on the exposed X-ray films or in the phosphoimager-processed files.
13. PTK/PTP expression profiles can be established by the radiation dose unit numbers of different kinase/phosphatase identified. (*see* **Note 6**.)

4. Notes

1. The catalytic domains of PTK and PTP can be divided into a number of smaller subdomains that represent localized regions of high conservation ***(30,31)***. By using degenerate primers from these regions, as well as the RT-PCR strategy, we could easily amplify most PTK/PTP genes expressed in cells, if not all PTKs/PTPs. The first PTK/PTP profiling method (*see* **Subheading 3.2.** protocol) required cloning and sequencing hundreds of clones following the RT-PCR reactions ***(16,25)***. Although it was an effective approach, it was time consuming for sequencing hundreds of clones. In addition, this protocol could not generate a more comprehensive and quantitative PTK/PTP profile until thousands of clones were sequenced. However, it is suitable for screening novel PTK/PTP genes in organisms without comprehensive genome sequence information as previously demonstrated by others ***(18,21,23)***.
2. In this second PTK/PTP display approach (*see* **Subheading 3.5.** protocol), the expression level of PTK kinase genes could be represented by the intensity of each recognized band. This protocol is established on the bioinformatic restriction digest databases, which require the sequences of almost all PTKs/PTPs. Otherwise, there will be many unknown fragments on the gel and thus severely diminishes the effectiveness of this display approach. Although one can excise the fragments of interest from gels and identify them by cloning methods ***(32)***, it is time consuming and less effective. Because it did not employ the previously used cloning and screening procedures, this display method can be quantitative and more representative by utilizing the radio-labeled primers ***(7,29)***.
3. Because the radioactive label is only on the forward primers, each radioactive signal is generated from one labeled primer. Another advantage of this method is that one can perform multiple PTK profiles on several cell lines or tissues at the same time, wherease it would be difficult to perform large amounts of screening with the previous cloning and sequencing method. As discussed before, a comprehensive database is essential for this display approach, which might be useful for human or mouse PTK/PTP profiling. One has to screen the genome sequence or EST databases to generate the restriction digest databases ***(12)***. We have now implemented bioinformatic programs to automatically screen the genome and generate restriction digest databases for display purposes. Another issue that could be resolved by bioinformatic tools is single nucleotide polymorphism (SNP) within the PTKs/PTPs.

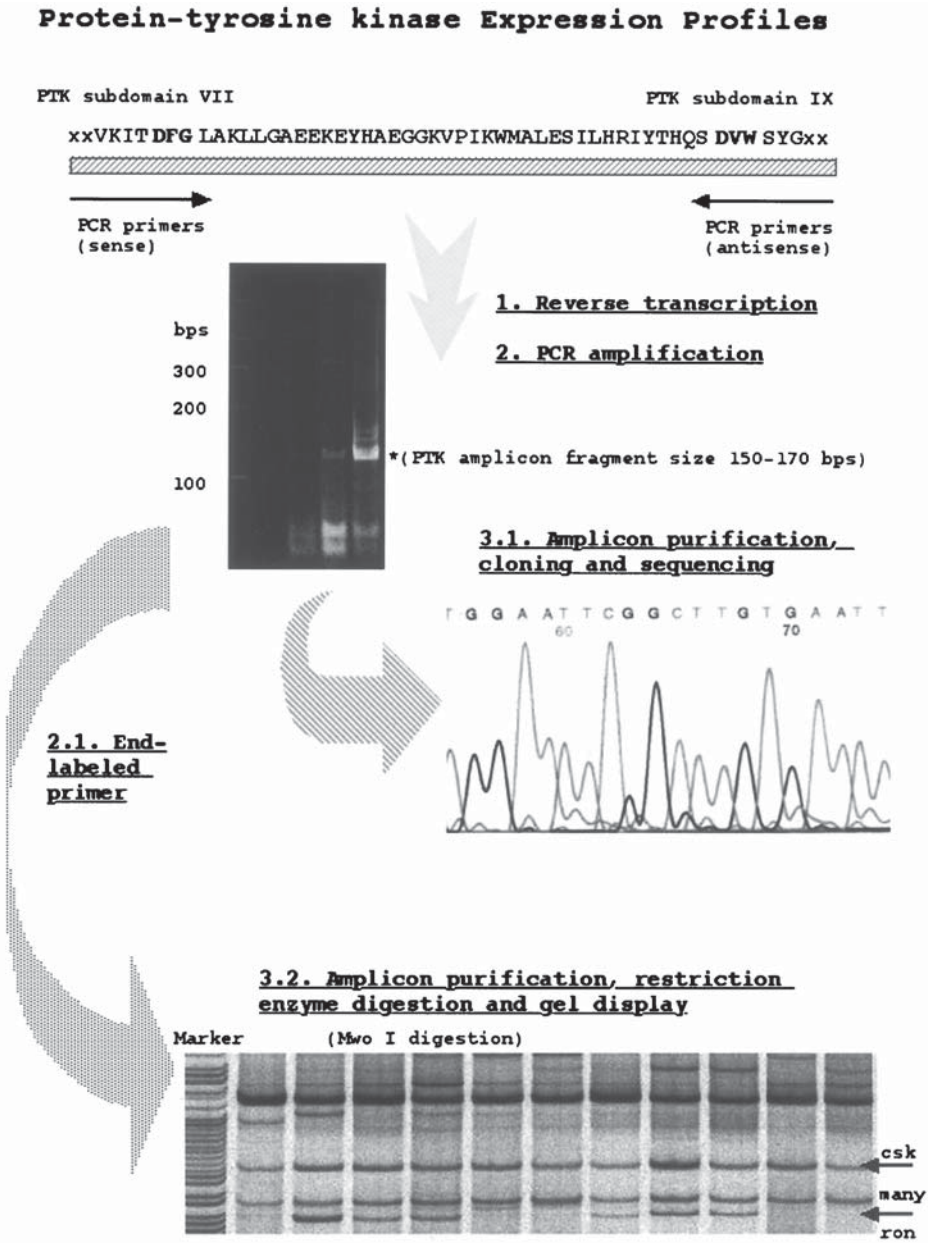

Fig. 1. Illustration of the PTK profiling approach. The detailed protocols are described in the Materials and Methods sections under corresponding subheadings.

4. Because the display approach depends on the restriction enzyme recognition, SNPs located in the amplified PTK/PTP regions will affect the restriction enzyme recognition and generate different digestion fragments. This is similar to restriction fragment length polymorphism. Generally, it is not an important issue for the PTK/PTP display approach, because we are using a panel of restriction enzymes to determine the identity of each PTK/PTP gene, not just one enzyme. In addition, similar size restriction fragments could represent several different kinases, and alternative restriction enzymes would be necessary to identify single gene specific restriction fragments. It was calculated that about 15–20 restriction enzymes (4-base or 5-base hitters) were required to cover all genes here. With the aid of bioinformatic software, we might be able to include SNP information in the databases in order to generate a better PTK/PTP profile. With completion of the human genome project, we could examine the entire human transcriptome and learn more about the composition and evolution of gene families in the human genome. This will greatly assist this display approach, because all restriction fragments or patterns could be assigned to every known gene.
5. Additional improvements can be achieved in primer design. We use the degenerate primers based on the conservative motifs of PTKs/PTPs to cover all known and unknown genes, and specificity is compromised with the degenerate primer design. Because we now have all the human genome sequences, new human profile primers can be designed to increase specificity and reduce cross-hybridization with other serine/threonine kinases or phosphatases.
6. One major drawback for this display protocol is the short half-life of radioactive isotope labeled primers. In the future, with the addition of fluorescent-labeled PCR primers, we can also include internal M.W. markers, control genes tagged with different color dyes in the same reaction. This will extend the usage of labeled primers, because there is no isotope half-life and relabeling considerations. Quantitation of each band was easier and less time consuming with fluorescent-labeled primers. With ^{33}P labels, exposure time to film or phosphoplate ranged from three to seven days. ^{32}P can be used to reduce the process time ***(32)***, however, gel resolution is much reduced owing to the strong ^{32}P radioactivity. Analysis could be performed immediately following gel separation with fluorescent labels on auto-sequencers.

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References

1. Hunter, T. (1995) Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. *Cell* **80,** 225–236.

2. Hunter, T. and Cooper, J. A. (1985) Protein-tyrosine kinases. *Annu. Rev. Biochem.* **54,** 897–930.
3. Hunter, T. (1987) A thousand and one protein kinases. *Cell* **50,** 823–829.
4. Kairouz, R. and Daly, R. J. (2000) Tyrosine kinase signalling in breast cancer: modulation of tyrosine kinase signalling in human breast cancer through altered expression of signalling intermediates. *Breast Cancer Res.* **2,** 197–202.
5. Lin, W., Kao, H. W., Robinson, D., Kung, H. J., Wu, C. W., and Chen, H. C. (2000) Tyrosine kinases and gastric cancer. *Oncogene* **19,** 5680–5689.
6. Weiner, H. L. and Zagzag, D. (2000) Growth factor receptor tyrosine kinases: cell adhesion kinase family suggests a novel signaling mechanism in cancer. *Cancer Invest.* **18,** 544–554.
7. Kung, H. J., Chen, H. C., and Robinson, D. (1998) Molecular profiling of tyrosine kinases in normal and cancer cells. *J. Biomed. Sci.* **5,** 74–78.
8. Tonks, N. K. (1996) Protein tyrosine phosphatases and the control of cellular signaling responses. *Adv. Pharmacol.* **36,** 91–119.
9. Sun, H. and Tonks, N. K. (1994) The coordinated action of protein tyrosine phosphatases and kinases in cell signaling. *Trends Biochem. Sci.* **19,** 480–485.
10. Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S. I., et al. (1997) PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science* **275,** 1943–1947.
11. Qiu, Y., Ravi, L., and Kung, H. J. (1998) Requirement of ErbB2 for signalling by interleukin-6 in prostate carcinoma cells. *Nature* **393,** 83–85.
12. Robinson, D. R., Wu, Y. M., and Lin, S. F. (2000) The protein tyrosine kinase family of the human genome. *Oncogene* **19,** 5548–5557.
13. Strausberg, R. L. (2001) The Cancer Genome Anatomy Project: new resources for reading the molecular signatures of cancer. *J. Pathol.* **195,** 31–40.
14. Velculescu, V. E., Zhang, L., Vogelstein, B., and Kinzler, K. W. (1995) Serial analysis of gene expression. *Science* **270,** 484–487.
15. DeRisi, J., Penland, L., Brown, P. O., Bittner, M. L., Meltzer, P. S., Ray, M., et al. (1996) Use of a cDNA microarray to analyse gene expression patterns in human cancer. *Nat. Genet.* **14,** 457–460.
16. Robinson, D., He, F., Pretlow, T., and Kung, H. J. (1996) A tyrosine kinase profile of prostate carcinoma. *Proc. Natl. Acad. Sci. USA* **93,** 5958–5962.
17. Venter, J. C., et al. (2001) The sequence of the human genome. *Science* **291,** 1304–1351.
18. Wilks, A. F., Kurban, R. R., Hovens, C. M., and Ralph, S. J. (1989) The application of the polymerase chain reaction to cloning members of the protein tyrosine kinase family. *Gene* **85,** 67–74.
19. Tanaka, K., Nagayama, Y., Nakano, T., Takamura, N., Namba, H., Fukada, S., et al. (1998) Expression profile of receptor-type protein tyrosine kinase genes in the human thyroid. *Endocrinology* **139,** 852–858.
20. Wilks, A. F. (1989) Two putative protein-tyrosine kinases identified by application of the polymerase chain reaction. *Proc. Natl. Acad. Sci. USA* **86,** 1603–1607.

21. Mossie, K., Jallal, B., Alves, F., Sures, I., Plowman, G. D., and Ullrich, A. (1995) Colon carcinoma kinase-4 defines a new subclass of the receptor tyrosine kinase family. *Oncogene* **11,** 2179–2184.
22. Takahashi, T., Shirasawa, T., Miyake, K., Yahagi, Y., Maruyama, N., Kasahara, N., et al. (1995) Protein tyrosine kinases expressed in glomeruli and cultured glomerular cells: Flt-1 and VEGF expression in renal mesangial cells. *Biochem. Biophys. Res. Commun.* **209,** 218–226.
23. Schultz, S. J. and Nigg, E. A. (1993) Identification of 21 novel human protein kinases, including 3 members of a family related to the cell cycle regulator nimA of Aspergillus nidulans. *Cell Growth Differ.* **4,** 821–830.
24. Lu, J., Li, Q., Donadel, G., Notkins, A. L., and Lan, M. S. (1998) Profile and differential expression of protein tyrosine phosphatases in mouse pancreatic islet tumor cell lines. *Pancreas* **16,** 515–520.
25. Lin, J. S., Lu, C. W., Huang, C. J., Wu, P. F., Robinson, D., Kung, H. J., et al. (1998) Protein-tyrosine kinase and protein-serine/threonine kinase expression in human gastric cancer cell lines. *J. Biomed. Sci.* **5,** 101–110.
26. Chen, W. S., Kung, H. J., Yang, W. K., and Lin, W. (1999) Comparative tyrosine-kinase profiles in colorectal cancers: enhanced arg expression in carcinoma as compared with adenoma and normal mucosa. *Int. J. Cancer* **83,** 579–584.
27. Lin, W. C., Li, A. F., Chi, C. W., Chung, W. W., Huang, C. L., Lui, W. Y., et al. (1999) tie-1 protein tyrosine kinase: a novel independent prognostic marker for gastric cancer. *Clin. Cancer Res.* **5,** 1745–1751.
28. Wu, C. W., Li, A. F., Chi, C. W., Huang, C. L., Shen, K. H., Liu, W. Y., and Lin, W. (2000) Human gastric cancer kinase profile and prognostic significance of MKK4 kinase. *Am. J. Pathol.* **156,** 2007–2015.
29. Meric, F., Lee, W. P., Sahin, A., Zhang, H., Kung, H. J., and Hung, M. C. (2002) Expression profile of tyrosine kinases in breast cancer. *Clin. Cancer Res.* **8,** 361–367.
30. Hanks, S. K., Quinn, A. M., and Hunter, T. (1988) The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science* **241,** 42–52.
31. Saito, H. (1993) Structural diversity of eukaryotic protein tyrosine phosphatases: functional and evolutionary implications. *Semin. Cell Biol.* **4,** 379–387.
32. Lee, W. P., Liao, Y., Robinson, D., Kung, H. J., Liu, E. T., and Hung, M. C. (1999) Axl-gas6 interaction counteracts E1A-mediated cell growth suppression and pro-apoptotic activity. *Mol. Cell Biol.* **19,** 8075–8082.

10

Association of Nonreceptor Tyrosine Kinase *c*-Yes with Tight Junction Protein Occludin by Coimmunoprecipitation Assay

Yan-Hua Chen and Qun Lu

Abstract

Immunoprecipitation is one of the most commonly used techniques to study protein–protein interaction in vivo. There are three major steps involved in an immunoprecipitation procedure: 1) lysis of the cells to make the antigen of interest available; 2) formation of the antibody–antigen complex by adding specific antibody; 3) separation and detection of the immune complex by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Both polyclonal and monoclonal antibodies can be used to immunoprecipitate the antigen. Polyclonal antibodies usually bind to multiple sites on the antigen and, therefore, are more efficient than monoclonal antibodies. The advantage of using monoclonal antibodies for immunoprecipitations is the specificity of the antibody–antigen interaction because monoclonal antibodies bind to only one epitope of the antigen. If the antigen of interest is tyrosine phosphorylated, and this tyrosine phosphorylation is under investigation, each step has to be handled with extra care to ensure success of the procedure. The buffer used to lyse the cells depends on the nature of the antigen, with more stringent conditions required for integral membrane proteins than for soluble cytosolic proteins. In either case, it is very important to add the protease inhibitors to the lysis buffer in order to prevent degradation of the antigen.

Key Words: Occludin; tight junctions; non-receptor tyrosine kinase *c*-Yes; tyrosine phosphorylation; immunoprecipitation.

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1. Introduction

Tight junctions are the most apical components of junctional complexes in epithelial and endothelial cells, and create a biological barrier to maintain the selectivity of paracellular permeability. Occludin is an integral membrane protein specifically localized at tight junctions and has been shown to be important for normal tight junction physiology ***(1–4)***. Our previous work has demonstrated that occludin is tyrosine phosphorylated when localized at tight junctions and tyrosine dephosphorylated when localized in cytoplasm ***(5)***. Tyrosine phosphorylation of occludin is therefore postulated to contribute to the regulation of tight junction assembly and function. Many attempts have been made to search for tyrosine kinase(s) that phosphorylate(s) occludin in vivo. Recently, we have found that *c*-Yes, a nonreceptor tyrosine kinase, forms a stable immunocomplex with occludin in epithelial cells. However, because occludin is an integral membrane protein that has a predicted tetraspanning membrane topology, special immunoprecipitation conditions are needed to ensure protein solubility and retain antigen–antibody binding capability. We have developed an immunoprecipitation protocol that was used successfully to detect the formation of stable complexes of occludin with *c*-Yes. Using this protocol, we were able to show that occludin interacts with the nonreceptor tyrosine kinase *c*-Yes and that tyrosine phosphorylation of occludin was tightly linked to tight junction formation in epithelial cells.

2. Materials

2.1. Reagents

1. Cell lysis buffer (RIPA buffer): 1% Triton X-100 (10% stock solution, 4ºC), 0.5% sodium deoxycholate (10% stock solution), 0.2% SDS (10% stock solution), 150 m*M* NaCl (4 *M* stock solution), 10 m*M* HEPES (1 *M* stock solution, pH 7.3, 4ºC), 2 m*M* ethylenediamine tetraacetic acid (EDTA) (0.5 *M* stock solution), 10 µg/mL each of chymostatin, leupeptin, and pepstatin A (a mixed 10 mg/mL stock solution in DMSO, –20ºC). To inhibit tyrosine phosphatases, add 2 m*M* sodium orthovanadate (100 m*M* stock solution, 4ºC), 10 m*M* sodium pyrophosphate (250 m*M* stock solution), and 20 m*M* sodium fluoride (1 *M* stock solution).

   This buffer should be freshly made each time from individual stock solutions before the experiment. Sodium orthovanadate is made from powder and can be reused within 2 wk.
2. Protein A- Sepharose (beads used if polyclonal antibody is the precipitating antibody) and Protein G- Sepharose (beads used if monoclonal antibody is the precipitating antibody): add 1 g beads to 1 mL PBS and rotate the beads at 4ºC overnight. Store the beads at 4ºC with 0.02% sodium azide.
3. Wash buffers:

a. High salt: 0.5 *M* NaCl, 10 m*M* Tris-HCl, pH 7.4, 10 µg/mL each of chymostatin, leupeptin, and pepstatin A.
b. Tris-HCl buffer: 10 m*M* Tris-HCl, pH 7.4, 10 µg/mL each of chymostatin, leupeptin, and pepstatin A.
4. SDS gel running buffer: 1.5 g Tris-Base, 7.2 g glycine, 2.5 mL 20% SDS, bring volume up to 500 mL with dH_2O.
5. Transfer blot buffer: 19.76 g glycine, 3.03 g Tris-Base, 1 g SDS, 200 mL MeOH, bring volume up to 1000 mL with dH_2O. Store at 4ºC.
6. TBS: 6.87 g Tris-HCl, 0.8 g Tris-Base, 9 g NaCl, bring volume up to 1000 mL with dH_2O.
7. Stripping solution: 100 m*M* β-mercaptoethanol, 2% SDS, and 62.5 m*M* Tris-HCl, pH 7.6, freshly made before use.
8. 2X SDS sample buffer (10 mL): 4 mL 10% SDS, 2 mL glycerol, 1.2 mL 1 *M* Tris-HCl (pH 6.8), 2 mL 1 *M* dithiothreitol (DTT, 200 m*M*), 0.8 mL dH_2O, 0.2% bromophenol blue.

3. Methods

3.1. Prepare Cell Lysates

1. Wash confluent MDCK II cell monolayer three times with PBS and add 0.8 mL RIPA buffer to each 100-mm Petri dish on ice (*see* **Note 1**).
2. Use the cell scraper to scrape cells off the dish and then transfer the cell lysate to a prechilled 1-mL Eppendorf tube. Vortex the tube for 10 s and let the tube sit on ice for 20 min (*see* **Note 2**).
3. Homogenize the cell lysate on ice by passing it 20 times through a 22-gage needle (use 1-mL syringe). Vortex the tube and sit on ice for 30 min.
4. Centrifuge the lysate at 15,000*g* for 20 min at 4ºC and transfer supernatant to a prechilled 1-mL Eppendorf tube. Measure the total protein concentration of each sample by BCA protein assay kit (Pierce, Rockford, IL) and adjust to 1 mg/mL concentration.
5. Add 40 µL prewashed protein-A or protein-G Sepharose beads to the cell lysate and rotate at 4ºC for 1 h (*see* **Note 3**).
6. Spin down the lysate containing the beads at 5000*g* for 2 min at 4ºC and take supernatant to a fresh prechilled 1-mL Eppendorf tube.

3.2. Immunoprecipitation: Formation of Antigen–Antibody Complex

1. Incubate the supernatants from **Subheading 3.1., step 6** with either polyclonal anti-occludin (2.5 µg/mL, Zymed, South San Francisco, CA) or monoclonal anti-*c*-Yes antibody (5 µg/mL, Transduction Laboratories, Lexington, KY) and rotate at 4ºC overnight.
2. Wash protein A- or protein G-Sepharose three times with PBS to remove sodium azide (*see* **Note 3**). Incubate the supernatants with either 40 µL prewashed protein

A-Sepharose (for occludin) or 50 µL protein G-Sepharose (for *c*-Yes) for an additional 2–3 h at 4ºC.

3. Spin down the supernatants at 5000*g* for 2 min at 4ºC. Discard the supernatants and save the beads. Wash the beads three times with 1 mL RIPA buffer, one time with 1 mL high salt (0.5 *M* NaCl), and one time with 1 mL Tris-HCl buffer (10 m*M* Tris-HCl, pH 7.4). Elute bound protein (antibody-antigen complex) from the beads in 40–50 µL 2X SDS sample buffer and ready for electrophoresis, or store at –20ºC for later use.

3.3. Electrophoresis and Immunoblotting

1. Open one package of precast 8–16% (or 12%) Tris-glycine gel (Novex, Carlsbad, CA) and take off the well comb from the top of the gel, as well as the white plastic seal from the bottom of the gel. Remove the solution from each well using a loading pipet and place the gel into a minigel electrophoresis apparatus. Fill up the inner chamber of the apparatus with SDS gel running buffer and fill the tank with about two inches of SDS gel running buffer (as long as the bottom of the gel is emerged in the buffer).
2. Boil the immunocomplex in SDS sample buffer for 5 min. Spin down in a microfuge for 2 min and then load supernatant onto the precast gel (can be loaded 15–25 µL per lane depending on the intensity of the signal). Use prestained molecular weight markers to localize the position of the proteins. Separate the samples under 130 V for one gel and 180 V for two gels.
3. Prepare a large tray filled with 500 mL ice-cold transfer blot buffer a few minutes before finishing the electrophoresis. Lay an open gel transfer cassette with the white side facing the bottom of the tray. Upon completion of the electrophoresis, turn off the power switch, and take the gel cassette out from the apparatus. Lay the gel cassette (well side up) on a flat surface and insert the Gel Knife into the narrow gap between the two plates of the cassette. Push up and down gently on the knife's handle to separate the plates. Discard the plate without the gel and allow the gel to remain on the other plate.
4. Place a wet foam sponge on the white side of the transfer cassette and then place a filter paper of the same size on top of the sponge. Cut a nitrocellulose membrane to the same size as the gel and place it on the filter paper. Remove the gel carefully from the plate and place it on the top of the nitrocellulose membrane. Then put another filter paper on the top of the gel and a wet foam sponge on the filter paper to make a sandwich cassette (*see* **Note 4**). Close this sandwich cassette and place cassette into the transfer apparatus with the black side of the cassette facing the black side of the apparatus. Fill the tank of the transfer apparatus with the transfer blot buffer from the large tray (*see* **Subheading 3.3., step 3**) and move this apparatus into the larger tray (*see* **Note 5**). Transfer is done under 220 mA for 2.5 h.
5. Disassemble the sandwich cassette and rinse the membrane with dH_2O. Block the membrane with 5% nonfat dried milk (2.5 g dry milk in 50 mL TBS) or 5% BSA (2.5 g BSA in 50 mL TBS) (*see* **Note 6**) plus 0.1% Tween-20 (500 µL 10% Tween-20 to 50 mL 5% milk or 5% BSA) for 1 h at room temperature or 4ºC overnight

with a gently shaking motion. The transfer buffer can be reused for several times as long as it does not form precipitates or grow fungus.

6. Incubate the membranes either with antioccludin (1:1000 dilution in 5% milk), anti-*c*-Yes (1:2500 dilution in 5% milk), or antiphosphotyrosine (1:300 dilution in 5% BSA, Santa Cruz, CA) antibody for 1.5 h at room temperature. After washing the membranes three times with blocking solution, incubate them with secondary antibody for 1 h at room temperature. The secondary antibody used for occludin was HRP-conjugated antirabbit IgG (1:2500 dilution, Promega, Madison, WI). For *c*-Yes and antiphosphotyrosine, the secondary antibody was HRP-conjugated antimouse IgG (1:2500 dilution, Promega, Madison, WI).
7. After washing three times with blocking solution, one time with TBS plus 0.1% Tween-20 and one time with TBS, incubate the blots with Enhanced Chemiluminescence solution (one part of solution A plus one part of solution B, Amersham, Arlington Heights, IL) for 1 min. Drain the excess solution on a Kimwipe tissue using blunt forceps, and then lay the blots on a piece of clear plastic wrap. Fold the wrap with no air bubble on the blots. Place the blots in film cassette and put a film on the top of the blots in the dark. Develop the film.
8. When necessary, the blots can be stripped in stripping solution for 30 min at 55ºC. Wash the blots three times in TBS containing 0.1% Tween-20 before reprobing with specific antibody (*see* **Note 7**).

4. Notes

1. It is very important to add the tyrosine phosphatase inhibitors (2 m*M* sodium orthovanadate, 10 m*M* sodium pyrophosphate, and 20 m*M* sodium fluoride) to the RIPA buffer if tyrosine phosphorylation of occludin will be detected after immunoprecipitation.
2. We found that the lysates cannot be frozen (even at –80ºC) and rethawed if the tyrosine phosphorylation of the antigen needs to be studied after the immunoprecipitation. Phosphorylation of tyrosine residues is very unstable and easily dephosphorylated during the freezing-thawing process.
3. To prewash the beads, take the necessary amount of protein-A and protein-G Sepharose beads from the original containers to the eppendorf tubes and spin down for 1 min. Remove the solution and add 1 mL PBS. Vortex the tubes and spin down for another minute. Remove the solution and add PBS (50% of the initial volume).
4. Gently press the sandwich by fingers from left to right to get rid of air bubbles between the gel and the membrane. It will greatly affect the protein transfer efficiency if there are air bubbles between the layers.
5. The transfer apparatus should be surrounded by ice or ice packs to reduce the heat generated by the current. Overheating will affect the result of the transfer.
6. It is very critical to use BSA when blotting with antiphosphotyrosine antibody because milk-containing buffers interfere with the phosphotyrosine antibody's binding ability.
7. For example, cell lysates treated with or without *c*-Yes inhibitor were immunoprecipitated with antioccludin antibody and then blotted with antiphosphotyrosine

antibody. It was found that occludin became tyrosine dephosphorylated in the cells treated with *c*-Yes inhibitor compared with those without treatment. In order to determine whether the same amount of occludin was immunoprecipitated in each condition, the same membrane was stripped and reprobed with antioccludin antibody.

References

1. Balda, M. S., Whitney, J. A., Flores, C., Gonzalez, S., Cereijido, M., and Matter, K. (1996) Functional dissociation of paracellular permeability and transepithelial electrical resistance and disruption of the apical-basolateral intramembrane diffusion barrier by expression of a mutant tight junction membrane protein. *J. Cell Biol.* **134,** 1031–1049.
2. Chen, Y.-H., Merzdorf, C., Paul, D. L., and Goodenough, D. A. (1997) COOH terminus of occludin is required for tight junction barrier function in early *Xenopus* embryos. *J. Cell Biol.* **138,** 891–899.
3. McCarthy, K. M., Skare, I. B., Stankewich, M. C., Furuse, M., Tsukita, S. H., Rogers, R. A., et al. (1996) Occludin is a functional component of the tight junction. *J. Cell Sci.* **109,** 2287–2298.
4. Wong, V. and Gumbiner, B. M. (1997) A synthetic peptide corresponding to the extracellular domain of occludin perturbs the tight junction permeability barrier. *J. Cell Biol.* **136,** 399–409.
5. Chen, Y.-H., Lu, Q., Schneeberger, E. E., and Goodenough, D. A. (2000) Restoration of tight junction structure and barrier function by down-regulation of the mitogen-activated protein kinase pathway in Ras-transformed Madin-Darby Canine Kidney cells. *Mol. Biol. Cell* **11,** 849–862.

11

Isolation of Novel Substrates Using a Tyrosine Kinase Overlay/*In Situ* Assay

Irwin H. Gelman

Abstract

Identifying substrates of receptor and non-receptor protein tyrosine kinases (PTK), and how phosphorylation of these substrates affects signaling and cytoskeletal pathways, has been a key step in understanding the role of PTK in differentiation, mitogenesis and oncogenesis. However, it has been difficult to distinguish substrates phosphorylated directly by PTK vs those phosphorylated by PTK-activated kinases. The following describes an *in situ*/overlay technique in which purified PTK (in our case, FAK) can be used to identify potential substrates from filter lifts of protein produced from a cDNA expression library.

Key Words: Protein tyrosine kinase; kinase; cDNA; expression library; kinase array; tyrosine phosphorylation; substrates; overlay assay; cloning; signal transduction.

1. Introduction

Since the identification of the *Src* oncogene as a protein tyrosine kinase (PTK) in 1980 ***(1)***, numerous groups have identified and characterized scores of receptor and nonreceptor PTKs, with an ultimate focus on their roles in normal and oncogenic cell signaling. The human genome contains 90 PTKs plus 5 pseudogenes, with 58 receptor-type classified into 20 subfamilies and 32 nonreceptor type classified into 10 subfamilies ***(2)***. Even within a subfamily, such as the *Src*-family PTK (*Src, Fgr, Fyn, Yes, Blk, Hck, Lck, Lyn*), several members

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are broadly expressed in many tissue types whereas others are expressed in specific tissues ***(3)***. Additionally, the creation of specific PTK-deficient mice showing few or no developmental defects has confirmed long-held views that many PTKs are redundant.

The so-called focal adhesion kinase (FAK) was isolated by several labs as a 125-kDa protein that was rapidly tyrosine phosphorylated after attachment of cells to extracellular matrix (ECM) proteins such as fibronectin ***(4–6)***. FAK is one member of a small PTK family, which includes Pyk2, however, unlike Pyk2, which is expressed mostly in hematopoietic cells, FAK expression is ubiquitous. Tyrosine phosphatase inhibitors cause an increase in the number of focal contacts per cell whereas tyrosine kinase inhibitors decrease their formation ***(7)***. This suggests that PTKs such as FAK are involved with the formation of adhesion complexes. The finding that FAK expression and activity are often increased in malignant cancers further suggests a role for FAK in promoting the oncogenic phenotype. FAK-deficient mice die early in embryogenesis (<E8.5) indicating a critical role in development, however, fibroblasts derived from FAK knockout mice ***(8)*** exhibit an increased frequency of focal complex formation, suggesting that FAK facilitates focal complex turnover. The possibility that FAK plays several roles in a dynamic cellular process was strengthened by recent data showing that FAK both helps translocate *Src* from perinuclear sites to focal adhesion complexes ***(9)***, but then is induced to degrade in a process requiring *Src* kinase activity ***(10)***; FAK degradation then directly leads to focal complex turnover and G1 → S progression.

A well-accepted yet incomplete model (reviewed in ***11***) suggests that interaction between transmembrane integrin proteins and ECM leads to FAK autophosphorylation at Tyr^{397}, producing a binding site for the SH2 domain of *Src*-family PTKs. The subsequent activation of *Src* leads to its autophosphorylation as well as phosphorylation of FAK at other sites such as Tyr^{925}. This, in turn, leads to a cascade of binding by SH2- and SH3-containing adaptor proteins that recruit a host of signaling and cytoskeletal proteins to the site typically referred to as the "focal adhesion complex." Many of the cytoskeletal proteins that associate with focal adhesions following interaction of integrins with ECM, such as vinculin, tropomyosin, α-actinin, talin, paxillin, ezrin, radixin, moesin, and zyxin, facilitate the attachment of actin-based cytoskeletal matrices to the focal complex sites (reviewed in ***12,13***). Following integrin activation, many of these cytoskeletal proteins become tyrosine phosphorylated and subsequently lose binding affinity for the actin cytoskeleton.

Whereas the kinase domains of PTKs are required to transduce specific signal cascades, many groups have endeavored to identify putative tyrosine kinase substrates as possible signal mediators. Substrates have been identified by direct

analysis, following purification with antiphosphotyrosine antibodies or as PTK-associated proteins. There are several major drawbacks in these methods including their inability to identify i) potential substrates expressed at low endogenous levels, ii) direct versus indirect substrates, and iii) substrates phosphorylated by PTKs with overlapping substrate specificities.

In regards to FAK, several potential substrates have been implicated such as paxillin, the p85 subunit of phosphoinositol 3-kinase, $p130^{CAS}$, and the cytoskeletal/nonmuscle isoform of α-actinin ***(14–16)***. Indeed, purified or immunoprecipitated FAK can phosphorylate paxillin in vitro ***(14,17)***. However, it is unclear whether FAK phosphorylates specific substrates in vivo or whether it simply facilitates the activation of *Src*-family and other related PTKs that, in turn, phosphorylate these substrates. The phosphorylation of many of these substrates is induced by the oncogenic form of *Src* encoded by Rous sarcoma virus, v-*Src*, suggesting that they can be direct substrates (reviewed in ***18***). This enigma is made more potent given the ability of FAK–/– mouse embryo fibroblasts to induce tyrosine phosphorylation of CAS and paxillin ***(8)***.

In order to identify potentially direct substrates of FAK, we adapted novel kinase overlay/*in situ* assays previously described for *Src* and MAP kinase-activated kinase-2 ***(19,20)*** with several modifications. Briefly, membranes containing expressed proteins from γ cDNA library made from 15-d embryonic mouse mRNA were first blocked, then incubated with partially purified, baculovirus-expressed FAK in a kinase/ATP buffer. After stripping off any associated FAK, potential substrates were identified by antiphosphotyrosine immunoblotting. After at least three rounds of plaque purification, false-positives—mostly representing tyrosine kinases capable of autophosphorylation—were identified by their ability to produce positive signals in the antiphosphotyrosine blots after incubation in buffer lacking FAK. Using this method, we identified paxillin as a bona fide in vitro FAK substrate.

2. Materials

All solutions were prepared from analytical-grade reagents and deionized double-distilled or ultrapurified filtered (Millipore; Bedford, MA) water. All reagents were purchased from Sigma (St. Louis, MO), unless otherwise indicated.

2.1. Expression and Purification of Baculovirus-Expressed FAK

1. High Five™ or Sf2 insect cells (Invitrogen Corp.; Carlsbad, CA) (*see* **Note 1**).
2. Grace's insect cell medium supplemented with 0.02 mg mL^{-1} gentimycin (GIBCO/Invitrogen).
3. Recombinant FAK baculovirus ***(14)*** (gift of Jin-Lin Guan, Cornell University) (*see* **Note 2**).

4. CMF-PBS (calcium- and magnesium-free phosphate-buffered saline [PBS]): 137 m*M* sodium chloride, 2.7 m*M* potassium chloride, 8.0 m*M* Na_2HPO_4, 1.4 m*M* KH_2PO_4 (pH 7.2).
5. NP-40 lysis buffer: 20 m*M* Tris-HCl (pH 7.4), 137 m*M* NaCl, 0.5 m*M* ethylenediamine tetraacetic acid (EDTA), 0.5 m*M* ethylene glycol-*bis* (β-aminoethyl ether) N,N, N'N'-tetraacetic acid (EGTA), 10% (v/v) glycerol, 1% (v/v) NP-40, 1 m*M* Na_3VO_4, 1 m*M* phenylmethylsulfonyl fluoride (PMSF), 0.05 TIU/mL aprotinin, 100 m*M* leupeptin (Roche Applied Sciences; Nutley, NJ), 1 m*M* benzamidine (*see* **Note 3**).
6. Low protein-binding 0.22 μm filters (Millipore).
7. Gel filtration buffer: 20 m*M* Tris-HCl (pH 7.4), 137 m*M* NaCl, 0.5 m*M* EDTA, 10% (v/v) glycerol, 1 m*M* DL-dithiotreitol (DTT), 1 m*M* benzamidine.
8. Superdex 200 sizing column (Pharmacia; Peapack, NJ), equilibrated with one column volume of deionized water and three column volumes of gel filtration buffer.
9. Storage buffer: 50 m*M* Tris-HCl (pH 7.4), 0.5 m*M* EDTA, 0.5 m*M* EGTA, 40% (v/v) glycerol, 1 m*M* DTT, 0.1 m*M* PMSF.
10. Rabbit polyclonal FAK antibody (Cat. #sc-558; Santa Cruz Biotechnology, Inc.; Santa Cruz, CA).

2.2. Kinase Overlay/In Situ Assay

1. cDNA expression library (e.g., Mouse 15-d Embryo 5'-STRETCH PLUS™ cDNA library in λTriplEx, BD Biosciences Clontech; Palo Alto, CA).
2. *Escherichia coli* (E. coli) strain XL1-Blue MRF'.
3. LB broth: 5 g of NaCl, 5 g of Yeast Extract (DIFCO), 10 g of Tryptone (DIFCO).
4. 100 mm petri dishes containing 30 mL per dish of LB plus 0.7% agar supplemented with 15 μg L^{-1} tetracycline.
5. NZY broth (per liter): 5 g of NaCl, 2 g of $MgSO_4 \cdot 6H_2O$, 5 g of Yeast Extract (DIFCO), 10 g of NZ Amine (casein hydrolysate, Sigma); autoclaved.
6. 150 mm Petri dishes containing at least 75 mL per plate of NZY plus 0.7% agar (DIFCO).
7. "Top agar": NZY both plus 0.7% agarose (Life Science Invitrogen), autoclaved.
8. 1 *M* $MgSO_4$ (100 X stock).
9. 20% (w/v) Maltose (100 X stock).
10. 137 mm nylon filters (Hybond™; Amersham Pharmacia Biotech).
11. 10 m*M* isopropyl-β-D-thiogalactopyranoside (IPTG).
12. TBST buffer: 10 m*M* Tris-HCl (pH 7.4), 150 m*M* NaCl, 0.1% Triton X-100).
13. Kinase buffer: Tri-buffered saline and Tween (TBST) containing 0.5 m*M* $MnCl_2$.
14. Blocking buffer: kinase buffer containing 3% bovine serum albumin (BSA) fraction V.
15. Stripping buffer: 62.5 m*M* Tris-HCl (pH 7.0), 2% sodium dodecyl sulfate (SDS), 100 m*M* 2-β-mercaptoethanol.

2.3. Antiphosphotyrosine Immunoblotting

1. TBS/Tween: 50 m*M* Tris-HCl (pH 7.4), 137 m*M* NaCl, 0.1% (v/v) Tween-20.
2. Blocking buffer: TBS/Tween plus 3% BSA.

3. Primary Ab solution: MAb-4G10 (Upstate Biotechnology, Inc.; Lake Placid, NY) at a 1:5000 dilution in blocking buffer (*see* **Note 4**).
4. Secondary Ab solution: horseradish peroxidase-labeled antimouse Ig (Chemicon; Temecula, CA) at a 1:5000 dilution in TBS/Tween.
5. Chemiluminescence detection reagent such as ECL (Amersham Pharmacia Biotech) or Lumi-Light™ (Roche Applied Sciences; Indianapolis, IN).

3. Methods

3.1. Expression and Purification of Baculovirus-Expressed FAK

1. High Five™ or Sf21 cells are seeded into spinner cultures at a density of 1×10^6 cells mL^{-1} in Grace's medium (500 mL total), and then infected 24 h later with virus at a multiplicity of infection (MOI) of 5 plaque-forming units (pfu) per cell. Optimal FAK expression (roughly 2 d postinfection) was detected by immunoblotting of cell lysates [RIPA lysates ***(21)*** containing 10 µg total protein per lane of a SDS/7.5% polyacrylamide gel] using PAb anti-FAK.
2. Collect infected cells by centrifugation for 10 min at 1000*g*, 4ºC.
3. Wash cells once in 50 mL CMF-PBS.
4. Lyse cells by incubating for 15 min in 8 mL of ice-cold NP-40 buffer, with periodic, mild vortexing, followed by Dounce homogenization.
5. Remove debris and clarify supernatant by centrifugation: 20 min at 35,000*g* at 4ºC.
6. Filter the supernatant through a low protein binding 0.22 µm filter.
7. In a cold room (4ºC), load the filtrate onto a prewashed Superdex 200 column (100 mL bed volume) preequilibrated with gel filtration buffer. Allow the filtrate to enter the matrix, then carefully layer gel filtration buffer on top. Continue to feed buffer to the top of the column while simultaneously collecting 5 mL fractions at the bottom.
8. Analyze column fractions for FAK content by immunoblotting with anti-FAK Ab (normalizing for protein content). Pool FAK-positive fractions.
9. Dialyze pooled fractions in at least 1 L of storage buffer. Freeze 100–250 µL aliquots at −80ºC, and store in-use aliquots at −20ºC for up to 3 mo.

3.2. Kinase Overlay/In Situ Assay

1. A single colony of *E. coli* XL-1 Blue MRF' (previously grown overnight on LB-tetracycline medium) is inoculated into 10 mL of LB broth, and grown overnight at 37ºC with vigorous agitation. The bacteria are collected by 10 min centrifugation at 10,000*g* at 4ºC, then resuspended in 10 mL of LB supplemented with $MgSO_4$ and maltose, and stored for up to 1 week at 4ºC.
2. The λcDNA library (2.4×10^5 total pfu) is incubated with 1.6 mL of XL-1 Blue MRF' at room temperature for 10 min, then mixed with 25 mL per plate of top agar supplemented with $MgSO_4$ and maltose, and then plated onto 8 prewarmed 150 mm plated containing NZY-agar.

3. Recombinant protein expression is induced after 4 h incubation at 37ºC by overlaying with nylon filters impregnated with 10 m*M* IPTG, and then incubating for an additional 6 h (*see* **Note 5**).
4. Mark each filter in three asymmetric spots by piercing with a 27-gauge needle dipped in India ink. Make sure to pierce well into the agar and wet the needle with ink before each piercing. This will facilitate filter identification and plaque orientation.
5. The filters are peeled off and washed extensively in TBST to remove debris and residual bacterial lawn, then equilibrated in 250-mL kinase buffer for 1 h at room temperature with gentle agitation (*see* **Note 6**).
6. The filters are incubated in blocking buffer (50 mL per filter) for 1 h at room temperature with gentle agitation.
7. The filters are then sealed in a plastic bag containing 10 mL per filter of kinase buffer supplemented with a one tenth volume of baculovirus FAK, 250 μ*M* ATP, and 100 μ*M* Na_3VO_4, and then incubated for 1 h at 30ºC (*see* **Note 7**).
8. Wash once briefly with kinase buffer.
9. Incubate filters with two 15 min washes of stripping buffer (50 mL per filter) at 50ºC.
10. Wash filters extensively with TBS/Tween until the smell of the mercaptoethanol from the stripping buffer is no longer detectable.

3.3. Antiphosphotyrosine Immunoblotting

1. Incubate filters in a sealed plastic bag for 30–60 min in TBS/Tween/BSA (10 mL per filter) at room temperature. Leave sufficient room within the bag to cut open and reseal the bag.
2. Add MAb-4G10 directly to bag at 1:5000 dilution, reseal the bag, and incubate for 1–2 h at room temperature with constant agitation (*see* **Note 8**).
3. Remove the filters and wash thrice for 15 min per wash with TBS/Tween (50 mL per filter) with constant, vigorous agitation.
4. Seal the filters in a plastic bag containing TBS/Tween/BSA supplemented with secondary Ab (10 mL per filter). Incubate for 1 h at room temperature with constant agitation.
5. Remove the filters and wash three times for 15 min per wash with TBS/Tween (50 mL per filter) with constant, vigorous agitation.
6. Wash once briefly with TBS to remove residual Tween-20 detergent.
7. Immerse filters in chemiluminescence solution, sandwich between layers of plastic wrap, and expose to film for 5–60 s or until individual positive plaques are identified (*see* **Fig. 1B**).
8. Re-orient the filters based on the India ink spots, and identify positive plaques.
9. Initially pick the positive plaques with the wider end of a sterilized Pasteur pipet. Subsequent rounds of plaque purification can be accomplished with the narrow end of the Pasteur pipet. Resuspend the plugs into 1 mL of LB supplemented with $MgSO_4$ and maltose, vortex vigorously, and then titer the phage (usually in the range of 10^4–10^6 pfu mL^{-1}).

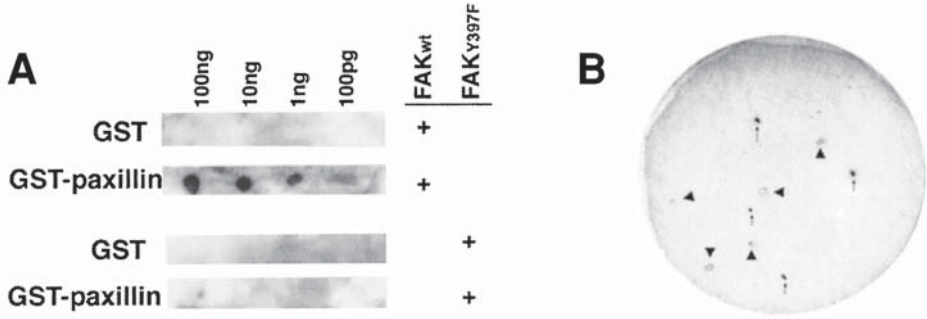

Fig. 1. (**A**) Functionality of the partially purified baculovirus-expressed FAK. To test the specificity and in vitro kinase activity of the baculovirus FAK, nitrocellulose strips were impregnated with various concentrations of either GST or GST-paxillin (gift of J. T. Parsons, University of Virginia). The strips were subjected to kinase overlay/*in situ* assay using either baculovirus FAK or FAK^{Y397F} (gift of Jun-Lin Guan) which lacks an autophosphorylation site, and thus, is deficient in intrinsic kinase activity (reviewed in ***22***). Note that i) only wt-FAK phosphorylates GST-paxillin and ii) the sensitivity of FAK (between 100 pg and 1 ng of target protein) is below the typical amount of recombinant protein expressed within a phage plaque (I. H. Gelman, unpublished data). (**B**) Positive plaques for FAK tyrosine phosphorylation (closed triangles) compared to India ink spots (arrows) used to orient membranes.

10. Repeat screening procedure until individual positive plaques are identified (*see* **Note 9**).
11. cDNA inserts of positive clones can be identified by directly sequencing the λ DNA or by rescuing plasmid forms with helper phage (*see* **Note 10**).

4. Notes

1. Roughly 20-fold more FAK is expressed in either High Five™ or Sf21 versus Sf9 cells ***(23)***. One advantage for using High Five™ or Sf21 (rather than mammalian) cells is that they do not express endogenous FAK ***(23)***.
2. Enzymatically active baculovirus-expressed FAK containing an N-terminal His6-tag has been reported ***(17)***.
3. PMSF stock solution (100X) is produced as a 100-m*M* solution in dimethylsulfoxide (DMSO), and must be added just before the buffer is to be used because its short half-life in aqueous solutions.
4. Antiphosphotyrosine MAb-PY20 (Transduction Laboratories) can also be used at a 1:10,000 dilution, although this often leads to a higher background.
5. Additional IPTG-impregnated nylon or nitrocellulose filters can be applied to the cells after the first filters are peeled off, although i) the second set tend to have lower recombinant protein yields per plaque and ii) positive plaques appear as halos rather than solid dark spots due to bacterial lysis in the center of the plaque.

6. In order to decrease the amount of bacterial lawn and debris that peels off with the filters, it is very important to cool the plates at 4ºC for at least 30 min prior to peeling.
7. An alternate method involves using [(γ-^{32}P]-ATP instead of cold ATP, followed by extensive washing with kinase stripping buffers, and then skipping the anti-phosphotyrosine immunoblotting step. A similar technique was used by Fukunaga and Hunter ***(20)*** in the process of cloning a novel MAP kinase-activating kinase. It should be noted that this technique yielded very high background, even if 3–5% BSA was included in all steps as a non-specific protein blocking agent. However, if attempted, it is advisable that prior to the actual kinase phosphorylation step, the filters should be incubated for 1 h at 30ºC in kinase buffer containing cold ATP (250 μM) and vanadate (100 μM) in order to saturate the autophosphorylation sites on all clones encoding tyrosine kinases.
8. The primary Ab solution can be reused three times within 1 wk if stored at 4ºC.
9. False-positive clones typically encode tyrosine kinases capable of autophosphorylation under the assay conditions used here. Once a set of signal-positive, purified plaques is identified, false-positives can be culled by performing the kinase overlay/*in situ* assay in kinase buffer containing only ATP and vanadate (no purified PTK), followed by antiphosphotyrosine blotting.
10. Using FAK as the PTK, we identified a 3' partial clone with 98% similarity at the nucleotide level to mouse paxillin (Genbank accession #BC003298).

References

1. Hunter, T. and Sefton, B. M. (1980) Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. *Proc. Natl. Acad. Sci. USA* **77,** 1311–1315.
2. Robinson, D. R., Wu, Y. M., and Lin, S. F. (2000) The protein tyrosine kinase family of the human genome. *Oncogene* **19,** 5548–5557.
3. Thomas, S. M. and Brugge, J. S. (1997) Cellular functions regulated by Src family kinases. *Ann. Rev. Cell Dev. Biol.* **13,** 513–609.
4. Guan, J. L., Trevithick, J. E., and Hynes, R. O. (1991) Fibronectin/integrin interaction induces tyrosine phosphorylation of a 120-kDa protein. *Cell. Regul.* **2,** 951–964.
5. Chan, P. Y., Kanner, S. B., Whitney, G., and Aruffo, A. (1994) A transmembrane-anchored chimeric focal adhesion kinase is constitutively activated and phosphorylated at tyrosine residues identical to pp125FAK. *J. Biol. Chem.* **269,** 20,567–20,574.
6. Lipfert, L., Haimovich, B., Schaller, M. D., Cobb, B. S., Parsons, J. T., and Brugge, J. S. (1992) Integrin-dependent phosphorylation and activation of the protein tyrosine kinase pp125FAK in platelets. *J. Cell. Biol.* **119,** 905–912.
7. Burridge, K. and Chrzanowska-Wodnicka, M. (1996) Focal adhesions, contractility, and signaling. *Ann. Rev. Cell Dev. Biol.* **12,** 463–519.
8. Ilic, D., Furuta, Y., Kanazawa, S., Takeda, N., Sobue, K., Nakatsuji, N., et al. (1995) Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature* **377,** 539–544.

9. Schaller, M. D., Hildebrand, J. D., and Parsons, J. T. (1999) Complex formation with focal adhesion kinase: A mechanism to regulate activity and subcellular localization of Src kinases. *Molev. Biol. Cell* **10,** 3489–3505.
10. Fincham, V. J. and Frame, M. C. (1998) The catalytic activity of Src is dispensable for translocation to focal adhesions but controls the turnover of these structures during cell motility. *EMBO J.* **17,** 81–92.
11. Schlaepfer, D. D., Hauck, C. R., and Sieg, D. J. (1999) Signaling through focal adhesion kinase. *Prog. Biophys. Mol. Biol.* **71,** 435–478.
12. Kumar, C. C. (1998) Signaling by integrin receptors. *Oncogene* **17,** 1365–1373.
13. Cary, L. A. and Guan, J. L. (1999) Focal adhesion kinase in integrin-mediated signaling. *Front. Biosci.* **4,** D102–D113.
14. Chen, H. C. and Guan, J. L. (1994) Association of focal adhesion kinase with its potential substrate phosphatidylinositol 3-kinase. *Proc. Natl. Acad. Sci. USA* **91,** 10,148–10,152.
15. Ruest, P. J., Shin, N. Y., Polte, T. R., Zhang, X., and Hanks, S. K. (2001) Mechanisms of CAS substrate domain tyrosine phosphorylation by FAK and Src. *Mol. Cell. Biol.* **21,** 7641–7652.
16. Izaguirre, G., Aguirre, L., Hu, Y. P., Lee, H. Y., Schlaepfer, D. D., Aneskievich, B. J., and Haimovich, B. (2001) The cytoskeletal/non-muscle isoform of α-actinin is phosphorylated on its actin-binding domain by the focal adhesion kinase. *J. Biol. Chem.* **276,** 28,676–28,685.
17. Withers, B. E., Keller, P. R., and Fry, D. W. (1996) Expression, purification and characterization of focal adhesion kinase using a baculovirus system. *Prot. Expr. Purif.* **7,** 12–18.
18. Brown, M. T. and Cooper, J. A. (1996) Regulation, substrates and functions of src. *Biochim. Biophys. Acta Rev. Cancer* **1287,** 121–149.
19. Lock, P., Abram, C. L., Gibson, T., and Courtneidge, S. A. (1998) A new method for isolating tyrosine kinase substrates used to identify Fish, an SH3 and PX domain-containing protein, and Src substrate. *EMBO J.* **17,** 4346–4357.
20. Fukunaga, R. and Hunter, T. (1997) MNK1, a new MAP kinase-activated protein kinase, isolated by a novel expression screening method for identifying protein kinase substrates. *EMBO J.* **16,** 1921–1933.
21. Lin, X., Tombler, E., Nelson, P. J., Ross, M., and Gelman, I. H. (1996) A novel *src*- and *ras*-suppressed protein kinase C substrate associated with cytoskeletal architecture. *J. Biol. Chem.* **271,** 28,430–28,438.
22. Guan, J. L. (1997) Role of focal adhesion kinase in integrin signaling. *Int. J. Biochem. Cell Biol.* **29,** 1085–1096.
23. Lacoste, J., Ma, A., and Parsons, J. T. (1998) Assay and purification of focal adhesion kinase. *Methods Enzymol.* **298,** 89–102.

12

Manipulating Expression of Endogenous Oncogenic Proteins Using an Antisense Oligonucleotide Approach in Prostate Cancer Cells

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Abstract

It has been shown that antisense oligodeoxynucleotide (ODN) treatments provide an effective, specific approach to inhibiting the function of target proteins. Using this method, we have acquired additional evidence that protein kinase C-epsilon functions as an oncogenic protein in the progression of recurrent human prostate cancer. This chapter describes the use of antisense ODN to directly target cellular protein kinase C-epsilon as a potential chemotherapeutic agent for blocking the advance of prostatic adenocarcinoma to androgen-independence. Using Lipofectin® as the carrier, phosphorothioate-modified antisense ODNs were transferred into prostate cancer cells with high efficiency, effectively inhibiting the expression of endogenous protein kinase C-epsilon and the androgen-independent (AI) proliferation of several independent human prostate cancer cell lines.

Key Words: Antisense ODN; prostate cancer; PKC-epsilon.

1. Introduction

Disordered signal transduction events that are permissive to unrestricted cell-cycle progression and escape from proapoptotic signals directly contribute to the malignant transformation of cancer cells ***(1,2)***. Gene transfer studies involving the use of constitutively active/inactive mutant signaling molecules have provided a wealth of information concerning the identities of putative oncogenic

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and tumor-suppressor proteins. However, stable transfections of these mutant constructs naturally lead to the induction of a complex series of direct and indirect adaptive responses in the cellular host. For this reason, alternative experimental approaches such as transient transfections, inducible expression vectors, and antisense oligodeoxynucleotides (ODN) have often been incorporated into investigations of aberrant cancer cell signaling molecules.

Various gene delivery approaches have been developed to introduce gene-specific DNA into target cells or organs using nonviral (plasmid DNA, DNA-coated gold particles, liposomes, and polymer DNA complexes) and viral (adenovirus, retrovirus, adeno-associated virus, herpes virus and pox virus) vectors/carriers. Each approach has its own strengths and weaknesses (for review, *see* **ref. 3**). Gene-specific antisense ODNs have proven to be effective in suppressing the malignant effects of aberrant cell signaling by oncogenic proteins in a variety of cancer cells. "Bystander" effects associated with these agents are minimal when compared to those induced by conventional chemotherapeutic agents because antisense constructs are capable of recognizing and downregulating the intended target without altering the expression of closely related members within the same gene family.

Protein kinase C (PKC) comprises a family of at least 11 closely related kinase isozymes ***(4)***. The family is conventionally subdivided into three main categories (classic, novel, and atypical PKCs) based on sequence homologies and cofactor requirements. PKC-epsilon (PKC-ε) was the first member of the novel PKC subfamily to be identified ***(5,6)*** and was subsequently shown to increase growth and cause malignant transformation when overexpressed in rodent fibroblasts by enhancing activation of Raf-1 kinase ***(7,8)***. PKC-ε is presently thought to be involved in the progression of various cancers, including cancers of the skin ***(9,10)***, brain ***(11)***, kidney ***(12)***, thyroid ***(13)***, blood ***(14)***, breast ***(15)***, colon ***(16)***, and prostate ***(17)***. Preliminary studies have established that the ectopic expression of PKC-ε is sufficient to transform LNCaP cells, a model of early-stage prostate cancer (CaP), into variants that are capable of establishing tumor growth in castrated mice. In clinical specimens of CaP, PKC-ε expression appears to be a reliable positive marker for the malignant epithelial cells that will survive androgen ablation therapy and be clonally selected during the recurrence of CaP. Thus, this oncogenic protein makes a conspicuous target for the adjunctive treatment of prostatic adenocarcinoma.

Our results to date show that downregulation of endogenous PKC-ε causes a block of cellular proliferation in vitro in androgen-independent (AI) CaP cell models (Wu, D., Terrian, D. M. [2002] *J. Biol. Chem.*, available online), providing a single gene-based therapeutic approach to CaP. Additional antisense ODNs that have been tested for their anti-tumor effects on CaP are listed in **Table 1**. In this chapter, we provide a detailed protocol using antisense ODN that is specifi-

Table 1
Some Genes Inhibited with Antisense ODNs in Prostate Cancer Model

Target	Tumor model/cell line	References
AR	LNCaP	***18***
Bcl-2	Shionogi tumor	***19***
		20
	LNCaP	***21***
	PC3	***22***
IGFBP-3	LNCaP	***23***
IGFBP-5	LNCaP	***24***
c-Raf	PC3	***25***
	DU145, PC3	***26***
		27
Metalloproteinase-7	LNCaP FGC	***28***
PAR	DU145	***29***
PKC-α	PC3	***22***
TRPM-2	PC3	***30***

cally targeted at PKC-ε to study cellular signaling in AI CaP cell lines DU145, PC3, and CWR-R1. However, the protocol provided below may be easily adopted for the study of alternative gene-specific targets.

2. Materials

2.1. Cell Culture

1. Prostate cancer cell lines DU145 and PC3 were obtained from the American Type Culture Collection (Manassas, VA; ATCC HTB-81 and CRL-1435, respectively). CWR-R1 was a generous gift from Dr. C W. Gregory (The University of North Carolina at Chapel Hill).
2. a) DU145 cells were cultured in Minimum essential medium Eagle with 2 m*M* L-glutamine and Earle's Balanced Salts Modified (BSS) adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 m*M* nonessential amino acids, 0.1 m*M* sodium pyruvate, and supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin. b) PC3 cells were cultured in F12K medium with 2 m*M* L-glutamine and supplemented with 10% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin. c) CWR-R1 cells were cultured in Prostate Growth Medium (PGM), comprised of Improved MEM Zinc Option (Richter's Improved MEM) supplemented with 900 µg/L linoleic acid, 1.2 g/L nicotinamide, 100 µg/L epidermal growth factor (EGF), 10 mg/L ITS (insulin/transferrin/selenious acid), 100 U/mL penicillin, 100 µg/mL streptomycin, and 2% FBS (31, and personal communication). Linoleic

acid and nicotinamide were from Sigma (St. Louis, MO). EGF and ITS were from Becton Dickinson Labware (Franklin Lakes, NJ). All other cell culture reagents were purchased from Invitrogen (Rockville, MD).
3. Phosphate buffered saline (PBS) and Trypsin-EDTA (10X) were purchased from Invitrogen.
4. Cell culture dish (150 mm), Corning, Inc., (Acton, MA).
5. Tissue culture plate (24-well), Becton Dickinson Labware.

2.2. Antisense Oligonucleotide Treatment

1. TE buffer: 10 m*M* Tris-HCl (pH 8.0), 1 m*M* ethylenediamine tetraacetic acid (EDTA).
2. Phosphorothioate-modified oligonuceotides, synthesized and purified at Invitrogen (*see* **Notes 1** and **2**), were prepared at concentrations of 50 µ*M* in 10 m*M* TE buffer under sterile conditions, and stored at –20ºC. The sequences are: antisense PKC-ε ODN (5'-CATGACGGCCGATGTGACCT-3') and scrambled PKC-ε ODN (5'-TACGCATAACGCGCTGGTGG-3') ***(32)***.
3. OPTI-MEM® I Reduced Serum Medium (Cat. No. 31985) and transfection reagent Lipofectin® (Cat. No. 18292-011) were purchased from Invitrogen.
4. Sterile tubes (15 × 120 mm), Becton Dickinson Labware.

2.3. Lysis of Cells and Electroblotting of Proteins (33)

1. Polyacrylamide minigel electrophoresis apparatus (Bio-Rad, Richmond, CA, Mini-Protein II system) with electrophoresis power supply.
2. Hoefer TE-22 Mighty Small Transfer Tank (Pharmacia Biotech, Piscataway, NJ) with cold water circulator.
3. IEC HN-SII Clinical centrifuge (International Equipment Company, Needham Heights, MA).
4. Sonicator.
5. Microcentrifuge.
6. Heating block.
7. Nitrocellulose paper (NCP, Bio-Rad. Cat. No. 162-015).
8. Sponges, paper towels, and plastic boxes.
9. 15 mL tubes.
10. Microcentrifuge tubes with screw-on caps.
11. Protease inhibitor stocks: store all stocks at –20ºC.
    a. Aprotinin (10 mg/mL) in deionized water.
    b. Pepstatin A (10 m*M*) in dimethylsulfoxide (DMSO). Store as 20 µL aliquots.
    c. Leupeptin (10 m*M*) in distilled water.
    d. Ovalbumin (10 mg/mL) in deionized water. Store as 200 µL aliquots.
12. PMSF stock: 5 mg phenylmethylsulfonyl fluoride (PMSF) in 50 µL methanol. Make fresh just before use.
13. Lysis buffer: 50 m*M* Tris-HCl, pH 7.5, 10 m*M* β-mercaptoethanol, 1% (Octylphenoxy)polyethoxyethanol (IGEPAL CA-630), 100 µg/mL aprotinin, 2 µ*M* pepstatin, 10 µ*M* leupeptin, 100 µg/mL ovalbumin, 100 µg/mL PMSF.

14. Solution A: Trizma base (1.5 *M*, pH 8.9) and EDTA (8 m*M*). Store at 4ºC.
15. Solution B: Trizma base (0.5 *M*, pH 6.8) and EDTA (8 m*M*). Store at 4ºC.
16. Solution C: 30% acrylamide/*bis* solution (37.5:1) was purchased from Bio-Rad. Store at 4ºC.
17. Solution D: 10% sodium dodecyl sulfate (SDS). Store at room temperature.
18. Solution E: 1.5% (w/v) ammonium persulfate. Make fresh every day.
19. 2X Western stop solution: 60 m*M* Trizma base, (pH 6.8), 7.5% (v/v) glycerol, 0.01% (w/v) bromphenol blue, 100 m*M* dithiothreitol (DTT), and 2% (w/v) SDS. Store at −20ºC.
20. SDS-polyacrylamide gel electrophoresis (PAGE) molecular weight marker: Invitrogen BenchMark Prestained Protein Ladder (cat. no. 10748-010). Store at −20ºC.
21. Reservoir buffer: 25 m*M* Trizma base, 187 m*M* glycine, and 0.1% (w/v) SDS. Adjust to pH 8.3. Prepare in stock solutions of 2 L and store at 4ºC.
22. Transfer blot buffer: 25 m*M* Trizma base, 192 m*M* glycine, 20% (v/v) methanol. Store at 4ºC.

2.4. Immunoblot Detection of PKC-ε

1. Stir plate.
2. X-ray film (Kodak BioMax ML, cat. no. 178-8207) and film cassette.
3. Saran wrap®.
4. Trizma buffered saline (TBS): Dissolve 40 mL 1 *M* Trizma HCl, pH 7.4, and 3.6 g NaCl in 4 L deionized water. Store at room temperature. Chill prior to use for washing blots.
5. TBS-Tween: Add 500 µL polyoxyethylenesorbitan monolaurate (Tween-20) to 1 L TBS. Store at 4ºC.
6. 5% TBS blotto: Dissolve 5 g nonfat milk in 100 mL TBS. Store at 4ºC. Make fresh on the day of use.
7. Enhanced chemiluminescence (ECL) detection solution: Mix 40 mL of 0.1 *M* Trizma HCl, pH 8.5, 4 mL of 0.68 m*M* p-Coumeric acid, 80 µL of 100 mg/mL 5-amino-2, 3-dihydro-1,4-phthalazinedione (luminol) in a glass flask and cover with foil because of the light sensitivity of luminol. Add 400 µL 3% hydrogen peroxide once in the darkroom. Must be made fresh on day of use.
8. Primary antibody: Polyclonal rabbit antihuman PKC-ε antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).
9. Secondary antibody: Antirabbit horseradish-peroxidase-conjugated IgG was purchased from Amersham (Arlington Heighs, IL). Add 12.5 µL of the appropriate secondary antibody to 50 mL TBS blotto. Store at −20ºC.

2.5. Cell Viability Assay

1. 24-well tissue culture plates.
2. Hemacytometer.
3. Trypsin-EDTA (1X).
4. Trypan blue solution (0.4%) was from Sigma.

3. Methods

3.1. Antisense Oligonucleotide Treatment

The following protocol is to transfect the cells in one well of a 24-well tissue culture plate. For other applications (such as Western blotting), use plate(s) of different size, and adjust the reagents accordingly.

1. Culture cells (37ºC, 5% CO_2) on 150 mm tissue culture plate in corresponding medium to 60–70% confluence. Serum-starve cells overnight.
2. Aspirate medium; wash the cells once with PBS. Add 5 mL Trypsin-EDTA onto the cells. Incubate at 37ºC for 10 min.
3. Count the cell numbers using Trypan blue and a hemacytometer. Suspend cells at 10^5 cells/mL medium, and plate in 24-well plates at 1 mL per well. Culture for 24 h before oligonucleotide treatment (*see* **Note 3**).
4. Prepare transfection solution A (for each transfection): dilute 4–20 µL ODN stock (50 µ*M*) in 50 µL OPTI-MEM I reduced serum medium at room temperature.
5. Prepare transfection solution B (for each transfection): incubate 2–10 µL Lipofectin in 50 µL OPTI-MEM I medium at room temperature for 30 min.
6. Combine solution A and solution B, mix gently, and incubate at room temperature for 15 min (*see* **Note 4**).
7. Set two controls: OPTI-MEM I medium without Lipofectin and ODN, OPTI-MEM I medium with Lipofectin only.
8. Add an additional 900 µL of OPTI-MEM I medium to the solution from **steps 6** and **7**, giving final ODN concentrations of 200 n*M*–1 µ*M*. Mix gently.
9. Wash the cells twice with 1 mL OPTI-MEM I medium.
10. Overlay the ODN/Lipofectin mixture onto cells, and incubate at 37ºC for 6 h.
11. Aspirate ODN/Lipofectin complex, wash the cells twice with 1 mL OPTI-MEM I medium, then add fresh medium containing 10% FBS and appropriate concentrations of ODNs, but no Lipofectin. Incubate the cells at 37ºC for 18 h (*see* **Note 5**).
12. Aspirate medium, add fresh medium containing 10% FBS and appropriate concentrations of ODNs. Incubate the cells at 37ºC for a further 48 h.

3.2. Cell Viability Assay

Cellular proliferation can be assessed using either the MTT (thiazolyl blue) assay, or the number of viable cells may be counted using a hemacytometer and trypan blue staining, as follows. Cell count should be performed in triplicate in 24-well tissue culture plates.

1. Aspirate medium; wash the cells once with PBS. Add 1 mL Trypsin-EDTA onto the cells. Incubate at 37ºC for 10 min.
2. Take 50 µL Trypsin-treated cells and mix with 50 µL Trypan blue. Add to a hemacytometer. View under the microscope and count viable cells.

3.3. Lysis of Cells and Electroblotting (33)

The following protocol is for a 150 mm plate. For different plate sizes, adjust the reagents accordingly.

1. Aspirate medium; wash the cells once with PBS. Add 5 mL Trypsin-EDTA onto the cells (150 mm plate). Incubate at 37ºC for 10 min.
2. Transfer to a 15-mL tube and add 5 mL of medium containing FBS.
3. Sediment at 1000*g* for 5 min on IEC HN-SII clinical centrifuge.
4. Pipet off supernatant and discard.
5. Resuspend cell pellet in 1 mL PBS, and transfer to microcentrifuge tubes.
6. Sediment at full speed (12,500*g*) for 30 s on microcentrifuge. Discard the supernatant.
7. Resuspend cell pellet in 200–500 µL lysis buffer depending on pellet size.
8. Sonicate resuspended cell pellet for 10–20 s and incubate on ice for 30 min.
9. Centrifuge at 12,500*g* on microcentrifuge for 10 min at 4ºC.
10. Transfer supernatants to new microcentrifuge tubes. Remove 5–50 µL aliquot for protein concentration determination. Add one-half volume of 2X Western stop solution to remaining samples. Boil for 3–5 min. Run SDS-PAGE or freeze samples at –20ºC.
11. For Western blotting of PKC-ε, prepare 10% acrylamide separating gel (enough for four minigels): Add 3.75 mL solution A, 10.0 mL solution C, 14.45 mL deionized water, 0.2 mL solution D, 1.0 mL solution E, 25 mL *N, N, N′, N′*-tetramethylethylenediamine (TEMED). Swirl gently to mix. Do not add solution E and TEMED until ready to pour gels.
12. Prepare 5% acrylamide stacking gel (enough for four minigels): Add 2.5 mL solution B, 5.0 mL solution C, 11.3 mL deionized water, 0.25 mL solution D, 2.5 mL solution E, 25 µL TEMED. Swirl gently to mix. Do not add solution E and TEMED until ready to pour gels.
13. Pour the gel(s) and polymerize.
14. Load equal amounts of sample protein into wells. Load prestained molecular weight markers on the same gel.
15. Run the gel(s) at 200 V until lowest molecular weight marker reaches the bottom of the separating gel (approx 1 h).
16. Turn off power supply when SDS-PAGE is complete. Remove the gel from the glass plate and assemble the transfer sandwich consisting of the following layers: black side of gel cassette (negative); 6 mm foam sponge; blotter paper; gel; NCP; blotter paper; two 3-mm foam sponges; and gray side of gel cassette (positive). Run at 400 mA for 90 min.

3.4. Enhanced Chemiluminescence (ECL) Detection

1. Block the membrane with 50 mL TBS blotto for 1 h at room temperature.
2. Prepare the primary antibody for incubation with membrane. Mix appropriate primary antibody with TBS blotto at the recommended dilution. Incubate the blot in primary antibody diluted in TBS blotto overnight at 4ºC.

3. Wash the blot as follows: TBS for 10 min; TBS-Tween for 10 min; TBS-Tween for 10 min; TBS for 10 min; and TBS for 5 min.
4. Prepare secondary antibody conjugate for incubation with the membrane. Mix appropriate secondary antibody with TBS blotto at the recommended dilution. Incubate the blot in secondary antibody diluted in TBS blotto for 1 h at room temperature.
5. Wash the blot as in **step 3**.
6. Mix ECL detection solution. Add 3% H_2O_2 just before using in the dark room.
7. Drain excess buffer from the washed blot, and place it in a clean plastic box.
8. Add ECL detection solution. Incubate for precisely 1 min at room temperature.
9. Remove blot from detection reagent, drain excess reagent. Lay blot on Saran Wrap®, avoiding air pockets. Place in film cassette, keeping the smooth side up.
10. Notch film (Kodak BioMax ML) in same place as blot. Place piece of film on top of blot, matching notches and close the film cassette. Expose the film for 1 min and develop it immediately. Estimate exposure time of subsequent film(s) on the basis of signal strength. We found that 3 d of treatment with 1 μ*M* antisense ODN significantly reduced the endogenous expression of PKC-ε in AI CaP cell lines DU145, PC3, and CWR-R1 (data not shown).

4. Notes

1. Though it is suggested (as the authors will do) that the oligonucleotide used in antisense treatment should be highly purified, such as, by reverse phase high-performance liquid chromatography (HPLC) or PAGE, to assure the specificity of antisense ODN as well as to minimize the cellular toxicity, we found that the quality of cartridge-purified ODN was adequate for routine treatment of cells on 150 mm plates. Be sure to include those controls (sense, mismatch, or scrambled ODN) no matter which kind of purification method is selected.
2. Instead of using the "fully-phosphorothioated" oligonucleotide, we found that those ODNs only with modification to the four bases in the 5' most position and the three bases next to the last one in the 3' most position are stable and effective for most applications.
3. For successful transfection with ODN, cell density must be optimized, which may vary among cell types. For the three CaP cell lines (DU145, PC3, CWR-R1) used in this protocol, 40–60% confluency was optimal. Cell confluency should be maintained constant in experiments to get reproducible results.
4. ODN/Lipofectin complex may appear cloudy after mixture, but that will not affect transfection ***(34)***. If too much visible precipitate appears, try to incubate the mixture for a shorter time (5–10 min).
5. Generally, it is not suggested to treat the cells with phosphorothiotide ODN alone, because phosphorothiotides are very nonspecifically active at the cell membrane ***(35)***. However, we found that treatments with 1 μ*M* "naked" ODN for a further 66 h following a 6 h incubation in ODN/Lipofectin complex could more effectively downregulate the protein level of PKC-ε in cells, without significant difference in cellular morphology when compared to the procedure using only ODN/Lipofectin complex for 6 h.

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References

1. Croce, C. M. (1987) Role of chromosome translocation in human neoplasia. *Cell* **49,** 155–156.
2. Rabbits, T. H. (1994) Chromosomal translocation in human cancer. *Nature* **372,** 143–149.
3. Harrington, K. J., Spitzweg, S., Bateman, A. R., Morris, J. C., and Vile, R. G. (2001) Gene therapy for prostate cancer: current status and future prospects. *J. Urology* **166,** 1220–1233.
4. Hug, H. and Sarre, T. F. (1993) Protein kinase C isoenzymes: divergence in signal transduction? *Biochem. J.* **291,** 329–343.
5. Ohno, S., Akita, Y., Konno, Y., Imajoh, S., and Suzuki, K. (1988) A novel phorbol ester receptor/protein kinase, nPKC, distantly related to the protein kinase C family. *Cell* **53,** 731–741.
6. Hanks, S. K. and Hunter, T. (1995) Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *FASEB J.* **9,** 576–596.
7. Wang, Q. J., Acs, P., Goodnight, J., Blumberg, P. M., Mischak, H., and Mushinski, J. F. (1998) The catalytic domain of PKC-epsilon, in reciprocal PKC-delta and -epsilon chimeras, is responsible for conferring tumorgenicity to NIH3T3 cells, whereas both regulatory and catalytic domains of PKC-epsilon contribute to *in vitro* transformation. *Oncogene* **16,** 53–60.
8. Cacace, A. M., Ueffing, M., Philipp, A., Han, E. K., Kolch, W., and Weinstein, I. B. (1996) PKC epsilon functions as an oncogene by enhancing activation of the Raf kinase. *Oncogene* **13,** 2517–2526.
9. Marks, F. and Gschwendt, M. (1995) Protein kinase C and skin tumor promotion. *Mutat. Res.* **333,** 161–172.
10. Reddig, P. J., Dreckschmidt, N. E., Zou, J., Bourguignon, S. E., Oberley, T. D., and Verma, A. K. (2000) Transgenic mice overexpressing protein kinase C epsilon in their epidermis exhibit reduced papilloma burden but enhanced carcinoma formation after tumor promotion. *Cancer Res.* **60,** 595–602.
11. Sharif, T. R. and Sharif, M. (1999) Overexpression of protein kinase C epsilon in astroglial brain tumor derived cell lines and primary tumor samples. *Int. J. Oncol.* **15,** 237–243.
12. Engers, R., Mrzyk, S., Springer, E., Fabbro, D., Weissgerber, G., Gernharz, C. D., and Gabbert, H. E. (2000) Protein kinase C in human renal cell carcinomas: role in invasion and differential isoenzyme expression. *Br. J. Cancer* **82,** 1063–1069.
13. Knauf, J. A., Elisei, R., Mochly-Rosen, D., Liron, T., Chen, X. N., Gonsky, R., et al. (1999) Involvement of protein kinase Cepsilon (PKCepsilon) in thyroid cell death. A truncated chimeric PKCepsilon cloned from a thyroid cancer cell line protects thyroid cells from apoptosis. *J. Biol. Chem.* **274,** 23,414–23,425.

14. Mayne, G. C. and Murray, A. W. (1998) Evidence that protein kinase Cepsilon mediates phorbol ester inhibition of calphostin C- and tumor necrosis factor-alpha-induced apoptosis in U937 histiocytic lymphoma cells. *J. Biol. Chem.* **273,** 24,115–24,121.
15. Lavie, Y., Zhang, Z. C., Cao, H. T., Han, T. Y., Jones, R. C., Liu, Y. Y., et al. (1998) Tamoxifen induces selective membrane association of protein kinase C epsilon in MCF-7 human breast cancer cells. *Int. J. Cancer* **77,** 928–932.
16. Perletti, G. P., Concari, P., Brusaferri, S., Marras, E., Piccinini, F., and Tashjian, A. H. Jr. (1998) Protein kinase Cepsilon is oncogenic in colon epithelial cells by interaction with the ras signal transduction pathway. *Oncogene* **16,** 3345–3348.
17. Cornford, P., Evans, J., Dodson, A., Parsons, K., Woolfenden, A., Neoptolemos, J., and Foster, C. S. (1999) Protein kinase C isoenzyme patterns characteristically modulated in early prostate cancer. *Am. J. Pathol.* **154,** 137–144.
18. Eder, I. E., Culig, Z., Ramoner, R., Thurnher, M., Putz, T., Nessler-Menardi, C., et al. (2000) Inhibition of LNcaP prostate cancer cells by means of androgen receptor antisense oligonucleotides. *Cancer Gene Ther.* **7,** 997–1007.
19. Gleave, M. E., Miyake, H., Goldie, J., Nelson, C., and Tolcher, A. (1999) Targeting bcl-2 gene to delay androgen-independent progression and enhance chemosensitivity in prostate cancer using antisense bcl-2 oligodeoxynucleotides. *Urology* **54 (6A Suppl.),** 36–46.
20. Monia, B. P. (2000) Antisense as a novel therapy for cancer, in *Pharmaceutical Aspects of Oligonucleotides* (Couvreur, P. and Malvy, C., eds.), Taylor & Francis, London, England, pp. 243–262.
21. Leung, S., Miyake, H., Zellweger, T., Tolcher, A., and Gleave, M. E. (2001) Synergistic chemosensitization and inhibition of progression to androgen independence by antisense Bcl-2 oligodeoxynucleotide and paclitaxel in the LNCaP prostate tumor model. *Int. J. Cancer* **91,** 846–850.
22. Benimetskaya, L., Miller, P., Benimetsky, S., Maciaszek, A., Guga, P., Eaucage, S. L., et al. (2001) Inhibtion of potentially anti-apoptotic proteins by antisense protein kinase C-alpha (Isis3521) and antisense bcl-2 (G3139) phosphorothioate oligodeoxynucleotides: relationship to the decreased viability of T24 bladder and PC3 prostate cancer cells. *Mol. Pharmacol.* **60,** 1296–1307.
23. Boyle, B. J., Zhao, X. Y., Cohen, P., and Feldman, D. (2001) Insulin-like growth factor binding protein-3 mediates 1 alpha, 25-dihydroxyvitamin d ***(3)*** growth inhibition in the LNCaP prostate cancer cell line through p21/WAF1. *J. Urol.* **165,** 1319–1324.
24. Miyake, H., Pollak, M., and Gleave, M. E. (2000) Castration-induced up-regulation of insulin-like growth factor binding protein-5 potentiates insulin-like growth factor-I activity and accelerates progression to androgen independence in prostate cancer models. *Cancer Res.* **60,** 3058–3064.
25. Geiger, T., Muller, M., Monia, B. P., and Fabbro, D. (1997) Antitumor activity of a C-raf antisense oligonucleotide in combination with standard chemotherapeutic agents against various human tumors transplanted subcutaneously into nude mice. *Clin. Cancer Res.* **3,** 1179–1185.

26. Lau, Q. C., Brusselbach, S., and Muller, R. (1998) Abrogation of c-Raf expression induces apoptosis in tumor cells. *Oncogene* **16,** 1899–1902.
27. Monia, B. P. (1997) First- and second-generation antisense oligonucleotide inhibitors targeted against human c-raf kinase, in *Oligonucleotides as Therapeutic Agents* (Ciba Foundation Symposium), Wiley, Chichester, England, pp.107–123.
28. Davies, G., Jiang, W. G., and Mason, M. D. (2001) Matrilysin mediates extracellular cleavage of E-cadherin from prostate cancer cells: a key mechanism in hepatocyte growth factor-induced cell-cell dissociation and in vitro invasion. *Clin. Cancer Res.* **7,** 3289–3297.
29. Platica, M., Ivan, E., Chen, S., Holland, J. F., Gil, J., Mandeli, J., and Platica, O. (2001) Stable lower PAR expression decreased DU145 prostate cancer cell growth in SCID mice. *Prostate* **49,** 200–207.
30. Miyake, H., Chi, K. N., and Gleave, M. E. (2000) Antisense TRPM-2 oligodeoxynucleotides chemosensitize human androgen-independent PC-3 prostate cancer cells both in vitro and in vivo. *Clin. Cancer Res.* **6,** 1655–1663.
31. Gregory, C. W., Johnson, R. T., Mohler, J. L. Jr., French, F. S., and Wilson, E. M. (2001) Androgen receptor stabilization in recurrent prostate cancer is associated with hypersensitivity to low androgen. *Cancer Res.* **61,** 2892–2898.
32. Traub, O., Monia, B. P., Dean, N. M., and Berk, B. C. (1997) PKC-epsilon is required for mechano-sensitive activation of ERK1/2 in endothelial cells. *J. Biol. Chem.* **272,** 31,251-31,257.
33. Wescott, G. G., Manring, C. M., and Terrian, D. M. (1998) Translocation assays of protein kinase C activation, in *Methods in Molecular Medicine, vol. 22: Neurodegeneration Methods and Protocols* (Harry, J. and Tilson, H.A., eds.), Humana, Totowa, NJ, pp. 125–132.
34. Lipofectin® Reagent (Cat. No. 18292-011) Technical Bulletin, Life Technologies.
35. Takle, G. B. and Stein, C. A. (2000) Delivery of antisense oligonucleotides in vitro, in *Pharmaceutical Aspects of Oligonucleotides* (Couvreur, P. and Malvy, C., eds.), Taylor & Francis, London, England, pp. 201–212.

13

Measurements of Phospholipases A_2, C, and D (PLA_2, PLC, and PLD)

In Vitro Microassays, Analysis of Enzyme Isoforms, and Intact-Cell Assays

Julian Gomez-Cambronero, Joel Horwitz, and Ramadan I. Sha'afi

Abstract

In order to be properly divisible, the cell membrane has to be remodeled and intracellular membranes must be converted into a vesiculated state prior to mitosis. Phospholipases A_2, C, and D (PLA_2, PLC, and PLD) are involved in regulatory events of intracellular mitogen signaling pathways. We describe here three methods for comprehensively assaying those phospholipases: 1) in vitro microassays, in which a radiolabeled substrate is exogenously added to cell lysates to measure the enzyme activity(ies); 2) immunocomplex assays, in which immunoprecipitation with a specific antibody is performed in order to study the contribution of a particular isoform within a family of enzymes; and 3) intact-cell or in vivo assays, in which cells are labeled with a radioactive substrate until steady state is reached. The uniqueness of the in vitro microassay method described here for the first time is that it allows the measurement of, in parallel, the activities of three phospholipases utilizing aliquots derived from *the same* biological sample. The approach for immunoprecipitation described in this chapter can be extrapolated to the study of a large array of enzyme isoforms. Finally, the intact-cell assays allow for the accurate measurement of receptor-mediated activation in vivo.

Key Words: Phospholipase in vitro assay; PLA_2; PLC; PLD; immunocomplex enzymatic reaction.

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1. Introduction

Phospholipases have been studied in great detail and their role in cell signaling has been established in the last 20 years. A role for phospholipases in mitogenesis has been described in terms of the products of their enzymatic reactions. Lysophosphatidic acid (LPA) is produced by the action of a phosphatidic acid (PA)-specific phospholipase A_2 (PLA_2). LPA is a potent mitogen for quiescent fibroblasts and triggers GTP-dependent phosphoinositide breakdown ***(1)*** through a membrane receptor ***(2)***. A phosphatidylcholine-specific PLA_2 is responsible for the release of arachidonic acid in response to cell stimulation. Arachidonic acid (AA) is the precursor of biologically active eicosanoids such as prostaglandins, thromboxanes and leukotrienes of key importance in the body's inflammatory response ***(3)***.

Phosphatidylinositol 4,5-bisphosphate (PIP_2)-specific phospholipase C, associated to membrane G proteins (PLCβ), hydrolyzes PIP_2 to form 1,2-diacylglycerol (DAG) and inositol 1,2,5-trisphosphate (IP_3). DAG and IP_3-generated Ca^{2+} are well known agonists of protein kinase C (PKC) activation ***(4,5)***, and this phospholipid breakdown is one of the earliest key events in the regulation of various cell functions, including cell growth. A phosphatidylcholine-specific PLC, associated to tyrosine kinases, also releases DAG as well as phosphorylcholine. The latter compound is known to be accumulated in the cell during the activation of Raf-1 and mitogen-activated protein (MAP) kinase that follows to growth factor stimulation and mitogenesis ***(6,7)***. Phosphocholine in conjunction with sphingosine-1-phosphate can greatly stimulate MAP kinase activity ***(8)***.

Phosphatidic acid (PA) is synthesized by the combined action of PLC and a DAG kinase, as well as by a direct action of phospholipase D (PLD) on membrane phospholipids. PA induces a transient intracellular Ca^{2+} rise and has growth-factor like effects on cultured cells ***(9)***. The formation of substantial amounts of PA immediately before entry into mitosis is important for establishing a delay in the cell cycle at the G_2/M border brought about by exogenous ligand ***(10)***. There are alternative pathways for PLC and PLD in regulatory events of intracellular mitogen signaling ***(11)***. In order to be properly divisible, internal membranes must be converted into a vesiculated state prior to mitosis. Owing to its membrane-perturbing and fusogenic properties, an increased level of PA in specific membrane compartments may counteract this process and the cell is delayed in the G_2 phase ***(12)***. Activation of PLD by serum is observed during *Ras*-induced transformation in NIH3T3 fibroblasts ***(13)***. Finally, the other product of PLD action, choline or ethanolamine (depending upon the nature of the membrane phospholipid) can also have mitogenic effects. Particularly, ethanolamine analogs stimulate DNA synthesis by a mechanism not involving phosphatidylethanolamine synthesis ***(14)***.

2. Materials

2.1. Preparation of the Biological Sample

1. Hanks' Buffer: 10 m*M* HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), pH 7.4, 150 m*M* NaCl, 5 m*M* KCl, 0.5 m*M* $CaCl_2$, 0.5 m*M* $MgCl_2$, 5 m*M* dextrose.
2. PLA_2 buffer: 250 m*M* Tris HCl, pH 8.5 (3.93 g/100 mL ddH_2O).
3. PLC buffer: 20 m*M* Tris-HCl pH 7.4, 60 m*M* NaCl, 3 m*M* $CaCl_2$ (0.32 g Tris-HCl, 0.35 g NaCl, 0.044 g $CaCl_2$ in 100 mL ddH_2O).
4. PLD buffer: 5 m*M* HEPES, pH 6.6. Prepare fresh each time from stock (200 m*M* HEPES, pH 6.6) as follows: 50 µL stock + 1.95 mL ddH_2O.
5. Protease/phosphatase inhibitors (recipe for 5 mL): 5 µg/mL aprotinin (12.5 µL from 1 mg/0.5 mL stock); 5 µg/mL pepstatin A (12.5 µL from 1 mg/0.5 mL stock); 5 µg/mL leupeptin (12.5 µL from 1 mg/0.5 mL stock); 100 µ*M* sodium orthovanadate (4 µL from 10 mg/0.5 mL stock); 40 µ*M* PMSF (1 µL from 10 mg/0.5 mL stock). Stocks are kept at −20ºC.
6. Right before the experiment add the protease/phosphatase inhibitors to 5 mL each of the PLA_2, PLC or PLD buffers and keep on ice.

2.2. PLA_2 In Vitro Microassay

1. Calcium solution: 100 m*M* $CaCl_2$ (1.47 g/100 mL ddH_2O).
2. Dilution buffer: 5 m*M* Tris-HCl, pH 8.5.
3. Radiolabeled PC (*PC): phosphatidylcholine, L-α-1-stearoyl-2-arachidonyl (specific activity 200 Ci/mmol; 1mCi/mL) from American Radiolabeled Chemicals (ARC).
4. Cold PC: L-α-phosphatidylcholine-β-arachidonyl-γ-stearoyl (C20:4/C18), dissolved at the concentration of 10 mg/mL in chloroform:methanol (9:1, v:v).
5. Positive controls: PLA_2 from bovine pancreas (purified enzyme), 5.7 U/mg (stock: 10 mg dissolved in 0.3 mL dilution buffer, aliquot in 50 µL and freeze [activity is 3 U/50 µL]). PLA_2 from bee venom (purified enzyme), 1225 U/mg (stock: 1 mg dissolved in 1 mL dilution buffer aliquot in 50 µL and freeze [activity is 60 U/50 µL]).
6. "Dole's extraction medium": isopropanol, n-heptane, H_2SO_4 (80:20:0.06, vol/vol).

2.3. PLC In Vitro Microassay

1. Deoxycholate (DOC) solution: dissolve 20 mg DOC in 1 mL of PLC buffer.
2. Radiolabeled PC (*PC): L-3-Phosphatidylcholine, 1-stearoyl-2-[1-^{14}C]arachidonyl (specific activity 55 mCi/mmol; 25 µLCi/mL) from Amersham.
3. Cold PC: L-α-phosphatidylcholine.
4. Positive controls: PLC from *B. cereus* (purified enzyme); stock: dissolve 25 U in 100 µL PLC buffer; aliquot into 50 µL and freeze (12 U/50 µL).
5. Stopping solution: methanol:chloroform:10 N hydrochloric acid (20:10:0.2, vol/vol/vol).
6. Authentic standards: Three species of diacylglycerol (DAG): C14:0/C14:0-DAG; C16:0/C18:0-DAG; C18:0/C20:4-DAG, dissolved separately in chloroform/methanol (9:1, vol/vol) at the concentration of 10 mg/mL (*see* **Note 1**).

7. Solvent system for TLC chamber ("hydrophobic solvent system"): 208.2 mL benzene, 39 mL Cl_3CH and 8.5 mL methanol (*see* **Note 2**).

2.4. PLD In Vitro Microassay

1. 200 m*M* HEPES.
2. 5 m*M* HEPES, pH 6.6 (50 µL of 200 m*M* HEPES + 1.95 mL ddH_2O).
3. PC8: 20 mg dioctanoyl phosphatidylcholine (PC8)/mL chloroform.
4. Radiolabelel butanol: [^{3}H]butan-1-ol (0.6 mCi/mL) from American Radiolabeled Chemicals (ARC).
5. Positive controls: PLD from cabbage (purified enzyme); 0.25 U/µL (dissolve 500 U in 2 mL PLD buffer. Store aliquots at –20ºC).
6. Stopping solutions and related solvents: chloroform/methanol (1:2), chloroform/methanol (95:5, vol/vol), ethyl acetate, iso-octane, acetic acid, 1% perchloric acid.
7. Authentic standard for TLC: dissolve PBut in chloroform:methanol (9:1, vol/vol) at the concentration of 10 mg/mL.
8. Solvent system for TLC chamber ("PLD solvent system"): In a separatory funnel, shake 130 mL ethyl acetate, 20 mL iso-octane, 30 mL acetic acid, and 100 mL ddH_2O. Allow the biphase to form and settle. Next, discard the lower phase, pour upper phase into TLC chamber lined with #3 Whatman paper (18 cm × 21 cm).

2.5. Immunoprecipitation and Immunocomplex Enzyme Assays

1. 1 *M* HEPES 23.8 g/100 mL H_2O and pH 7.3.
2. "10XLB" (stock lysis buffer): 100 m*M* HEPES, pH 7.3, 10 m*M* ethylene glycol-*bis* (β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 1 m*M* phenylmethylsulfonyl fluoride (PMSF), 2.1 µ*M* sodium orthovanadate, 1 m*M* dithiothreitol (DTT), 10 µ*M* ammonium molybdate, 12 m*M* diisopropyl fluorophosphate (DFP), 100 m*M* p-nitro-phenyl-phosphate, 10 m*M* β-glycerophosphate, 5.5% Triton X-100, and 5 µg/mL each of leupeptin, aprotinin, and pepstatin A (*see* **Note 3**).
3. LB: (1 mL '10XLB' + 7 mL Hank's + 2 mL glycerol) prepared right before the experiment; keep on ice.
4. LB/BSA: Dissolve 10 mg of fatty acid free-BSA in 1 mL of LB.
5. Antirabbit IgG secondary Antibody ("2ry") linked to agarose beads (this comes as a slurry with 1 µL packed beads per each 2 µL of slurry) (*see* **Note 4**).
6. Primary ("1ry") Antibody (polyclonal anti-PLD2) (*see* **Note 4**).
7. LiCl wash: 100 m*M* Tris-HCl, pH 7.4, 400 m*M* LiCl.
8. NaCl wash: 10 m*M* Tris-HCl, pH 7.4, 100 m*M* NaCl, 1 m*M* ethylenediamine tetraacetic acid (EDTA).

2.6. Intact-Cell (In Vivo) Assays

2.6.1. Measurement of cPLA_2

1. Hanks' Buffer: 10 m*M* HEPES, pH 7.4; 150 m*M* NaCl, 5 m*M* KCl, 0.5 m*M* $CaCl_2$, 0.5 m*M* $MgCl_2$, 5 m*M* dextrose.

2. Lysis Buffer: 50 m*M* HEPES, pH 7.4; 250 m*M* sucrose, 1 m*M* EGTA, 1 m*M* PMSF, 1 m*M* sodium orthovanadate, 5 m*M* DTT, 20 μ*M* each of aprotinin, leupeptin, and pepstatin.
3. [^{3}H]AA (usually dissolved in ethanol).
4. Stopping solution: Hexane/isopropanol/0.1 N HCl (300:200:4; vol/vol).

2.6.2. Measurement of PLCβ

1. Carrier-free $H_2[^{32}P]O_3$ (specific activity 1 mCi/mL).
2. Hanks' Buffer (RPMI or any tissue culture media of choice).
3. Lipid extraction mixture: hexane/isopropanol/0.1 N HCl (300:200:4 by vol).
4. Hexane/isopropanol (ratio 3:2 by vol).
5. X-ray films (Kodak O-mat).

2.6.3. Measurement of PLD

1. [^{3}H]palmitic acid (specific activity 5 mCi/mL).
2. Earl's Balanced Solution (EBS), RPMI (or tissue culture media of choice).
3. Authentic standard: Phosphatidylpropanol (PProp) (25 mg/mL stock) from Avanti Polar Lipids (Alabaster, AL). Before applying to TLC plate prepare the following "standard mix": 280 μL PProp + 970 μL chloroform.
4. Materials listed in points 6-8 under **Subheading 2.4.**

3. Methods

Here, we describe comprehensive methods for assaying phospholipases A_2, C, and D (PLA_2, PLC, and PLD). These assays are divided into three major sections: i) in vitro microassays (*see* **Subheading 3.1.**), in which a radiolabeled substrate is exogenously added to cell lysates or sonicates to measure the enzyme activity(ies); ii) immunocomplex assays (*see* **Subheading 3.2.**), in which immunoprecipitation with a specific antibody is performed (that is immediately followed by the in vitro microassays alluded to before) in order to study the contribution of a particular isoform within a family of enzymes; and iii) intact-cell or in vivo assays (*see* **Subheading 3.3.**), in which cells are labeled with a radioactive substrate until steady state is reached and then the intact cells are challenged with appropriate stimuli.

3.1. In Vitro Microassays

The uniqueness of the method described here is that it allows the measurement of, in parallel, the activities of three phospholipases utilizing aliquots derived from the *same* biological sample (**Fig. 1**). The biological sample is prepared as described in detail in **Subheading 3.1.1.** The cell sonicates generated are then split in three equal aliquots and are utilized for the in vitro microassay methods. These are tailored to the measuring of total activity for: PLA_2 (*see* **Subheading 3.1.2.**), PLC (*see* **Subheading 3.1.3.**) and PLD (*see* **Subhead-**

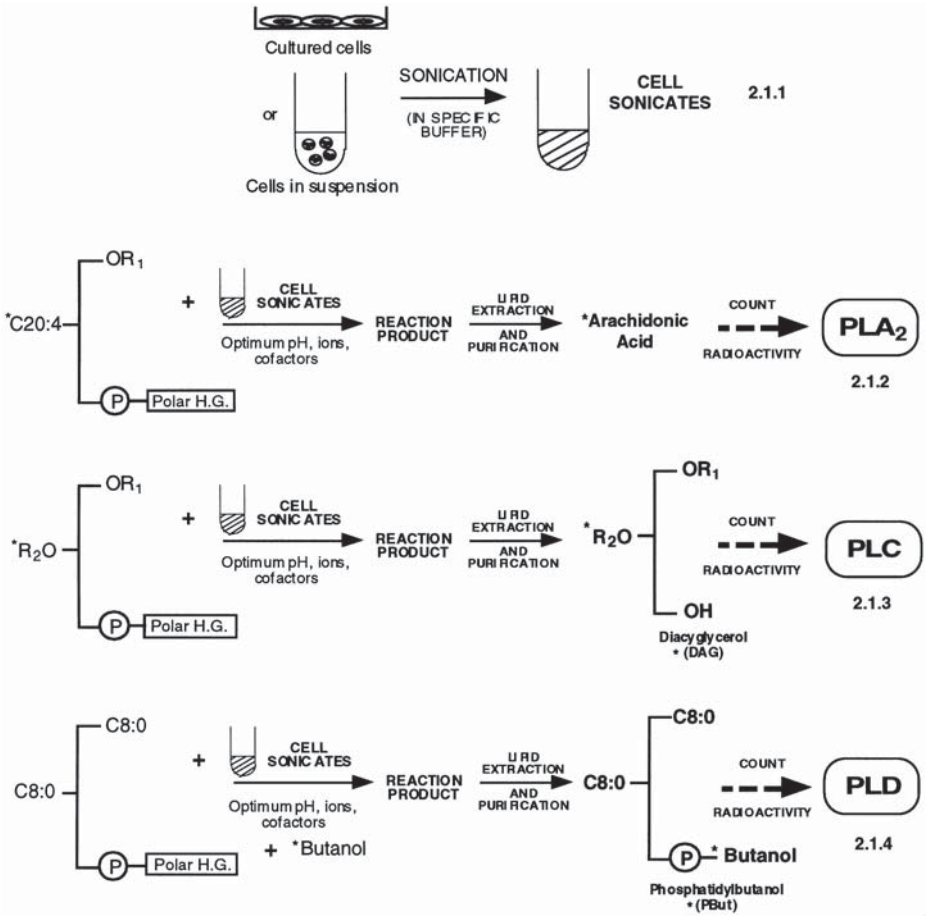

Fig. 1. In vitro microassays. From a common step that generates a "cell sonicate" mixture, the three phospholipases (PLA_2, PLC, and PLD) can be measured. The cell sonicates are incubated with the appropriate substrate (radiolabeled) and the product of the reaction is purified (normally by thin layer chromatography, TLC). Counting radioactivity at the end of the experiment provides cpm, that are a direct index of the phospholipase activity studied. (R1 and R2, generic fatty acid moieties in *sn*-1 and *sn*-2 positions; C20:4, arachidonoyl; C8:0, octanoyl; polar H. G., polar head group).

ing 3.1.4.). The specificity in the method for each phospholipase is conferred by the proper use of a specific substrate, reaction assay buffers and methods of isolation of enzymatic products. All the assays described below are "microassays," i.e., they can be performed in the laboratory using 1.5-mL Eppendorf tubes for all the reactions (instead of the traditional large glass test tubes). The small

reaction volumes allow for a saving in reagent expenses and processing time. The inclusion of positive controls (like purified enzymes), with known specific activities, is necessary for completeness ***(15)***.

3.1.1. Preparation of the Biological Sample, or "Source of Enzyme"

The very first step in the in vitro microassay protocols is the preparation of the sample from which the three enzyme activities (PLA_2, PLC, and PLD) will be measured.

1. Resuspend cells in fresh RPMI (culture cells) or Hanks' buffer (neutrophils) in 50 mL conical tubes (blue cap) at a concentration of $0.6 - 1 \times 10^7$ cells/mL (lower range for cultured cells; higher for neutrophils or any other small size cell).
2. Preincubate with stimuli if needed (*see* **Note 5**).
3. Take 1 mL aliquots in triplicate and transfer to prechilled eppendorf tubes kept on ice.
4. Spin down tubes at 1,744*g* for 30 s and decant supernatant.
5. Resuspend pellets in 1 mL of each buffer (PLA_2, PLC, or PLD) supplemented with the protease/phosphatase inhibitors (the volume should equate to approximately 500 µL buffer/mg protein).
6. Sonicate samples on ice (set sonicator at mid-high setting, 2 cycles, 10 s per cycle) (*see* **Note 6**).
7. Do a quick (30 s) spin (14,000*g*) to pull down unbroken cells and other debris. Transfer supernatants to clear eppendorfs labeled "cell sonicates" and leave on ice until ready to assay. Measure protein concentration (the yield is usually 1–2 mg/mL). Fifty microliters of these sonicates will be needed (per condition) for the in vitro microassays.

3.1.2. PLA_2 In Vitro Microassay

The first enzymatic assay we will consider is the measurement of PLA_2 activity in cell sonicates. The rate limiting step in eicosanoids synthesis is the release of arachidonic acid. This release involves PLA_2 activation and translocation to the compartments where the substrate (e.g., phosphatidylcholine), is located ***(16–23)***. The PLA_2 enzyme system is also crucial in the generation of the etherlipid platelet-activating factor (PAF), a mediator of inflammation. The cytosolic form of PLA_2 (*c*PLA_2), mediates the production of agonist-induced arachidonic acid release, and activation of *c*PLA_2 requires the phosphorylation of the enzyme, an increase in the concentration of intracellular free calcium, and the translocation to the compartments where the substrates are localized **(Fig. 2)**.

For in vitro measurements of PLA_2, the exogenous substrate is a phosphatidylcholine (PC) radiolabeled in the *sn*-2 position (AA). The radiolabeled AA (free fatty acid) released after the enzymatic cleavage is purified by TLC. The method indicated below will detect *total* PLA_2 activity present in the biological sample. For a specific analysis of cytosolic PLA_2 (*c*PLA_2) ***(24)*** (*see* **Note 7**).

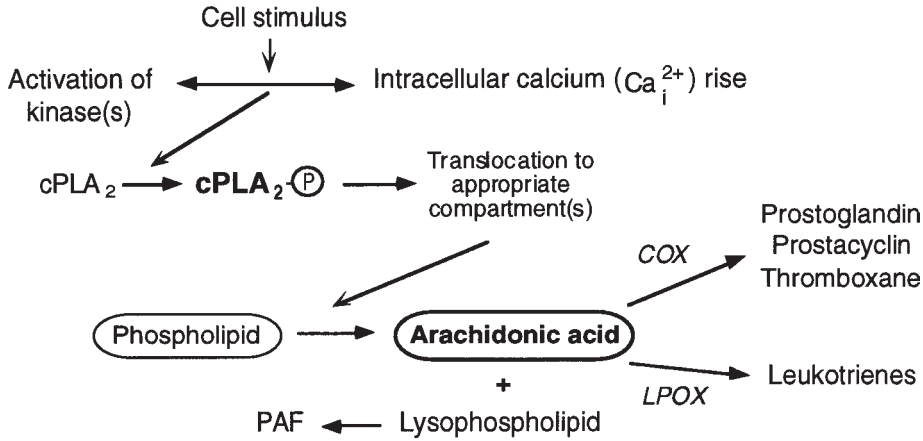

Fig. 2. Schematic representation of activation of cytosolic PLA_2 ($cPLA_2$) and the metabolism of AA. Kinase(s) include MAP kinase (p42/44), p38 MAP kinase and calcium/calmodulin-dependent protein kinase II. ($cPLA_2$-P, phosphorylated, active form of $cPLA_2$; PAF, platelet-activating factor; COX, cycloxygenase(s); LPOX, lipoxoxygenase).

3.1.2.1. Preparation of Liposomes

1. Label a small unsiliconized glass test tube "liposomes"; rinse with ddH_2O and dry.
2. Add 0.2 µL (0.2 µCi) of *PC per condition to the tube.
3. Add 1 µL (12 nmol) of cold PC per condition to the tube.
4. Dry lipids under Nitrogen.
5. Once dry, add 45 µL of PLA_2 buffer and 5 µL calcium solution per condition (final reaction concentration in a 100 µL volume is: 112.5 m*M* Tris HCl, 5 m*M* $CaCl_2$).
6. Vortex vigorously; let sit for approx 10 min at room temperature.
7. Vortex again for 10 s; sonicate briefly (sonicator in mid-low setting, 3 cycles of 3 seconds each) on ice. Keep liposomes on ice until needed.

3.1.2.2. Reaction and Lipid Separation

At this point the enzyme (in cell sonicates) is ready to be mixed with the radiolabeled substrate in the optimal conditions of cofactors, ionic strength, pH, etc.

1. To 1.5 mL screw cap tubes, add 50 µL of "liposomes" (from **Subheading 3.1.2.1.**) and 50 µL of "cell sonicates" (*see* **Subheading 3.1.1.**).
2. Include positive controls (pancreas and bee venom PLA_2 pure enzymes) and negative controls (PLA_2 buffer instead of cell sonicates).
3. Incubate 25–30 min at 30ºC in a orbital shaking incubator at 60 rpm.
4. Stop the reaction by adding 500 µL "Dole's extraction medium" to each tube with a repeater pipette.

5. Add 400 µL heptane.
6. Add 300 µL ddH_2O.
7. Vortex tubes and spin down using micro-centrifuge (14,000*g*, 1 min).
8. Carefully remove *upper* layer with a pipet and transfer to a clean Eppendorf tube.

3.1.2.3. Isolation of the Reaction Product (Radiolabeled Arachidonic Acid)

This is based in the method by Van den Bosh et al. ***(25)*** and involves the use of column chromatography for the separation of radiolabeled arachidonic acid (also *see* **Note 8**).

1. Prepare a glass wool plugged Pasteur pipet containing 240 mg silica gel (70–230 mesh pore size) per condition.
2. Prepare a rack of scintillation vials each containing 4 mL scintillation cocktail.
3. Secure Pasteur pipet with a clamp and position tip of pipet directly over scintillation vial.
4. Apply 400 µL of sample upper phase (from **Subheading 3.1.2.2.**, last step) to the pipet and wait until it becomes wet.
5. Next, add 800 µL of diethyl ether (via a 1 mL "tuberculin" syringe) and collect eluate ($[^3H]$AA) directly in scintillation vial containing (4 mL) scintillation cocktail.
6. Repeat procedure for each sample, using a new Pasteur pipet each time.
7. Cap and vortex scintillation vials and count by scintillation spectrometry.

3.1.3. PLC In Vitro Microassay

For PLC the substrate is a radiolabeled PC (either *sn*-1 or *sn*-2 positions) and the product of the reaction (radiolabeled diacyl-glycerol, DAG) is separated by TLC developed in a "hydrophobic" solvent system.

3.1.3.1. Preparation of Liposomes

This is based in the method by Waite and Smith ***(26)*** tailored for polymorphonuclear neutrophils, but can be applied to a variety of cultured cells.

1. Label a small unsiliconized glass test tube "liposomes"; rinse with ddH_2O and dry.
2. Dry the tube.
3. Add 2.5 µL cold PC (320 µ*M* final) and 2 µL *PC (50 nCi or 8 µ*M* final) per condition.
4. Dry lipids under nitrogen.
5. Once dry, add 48 µL of PLC buffer per condition to the tube.
6. Next, add 2 µL of diluted DOC per condition to the tube (1 m*M* final).
7. Vortex vigorously; sonicate briefly (low-mid setting) on ice and keep liposomes on ice until needed.

3.1.3.2. Reaction and Lipid Separation

At this point the enzyme (in cell sonicates) is ready to be mixed with the radiolabeled substrate in the optimal conditions of cofactors, ionic strength, pH, etc.

1. To 1.5 mL screw cap tubes, mix 50 µL of "liposomes" (*see* **Subheading 3.1.3.1.**) and 50 µL of "cell sonicates" (*see* **Subheading 3.1.1.**).
2. Include positive controls (*B. cereus* PLC pure enzyme) and negative controls (PLC buffer instead of cell sonicates).
3. Incubate 10 min at 30ºC in a orbital shaking incubator at 60 rpm.
4. Stop the reaction by adding 200 µL of stopping solution to each tube with repeater pipet.
5. Add 75 µL of Cl_3CH.
6. Add 25 µL ddH_2O.
7. Cap and vortex each tube vigorously.
8. Centrifuge at 2000*g* for 2 min.
9. Transfer 120 µL of the *lower layer* (organic phase containing the lipids) to a new set of transfer tubes.

3.1.3.3. Isolation of the Reaction Product (Radiolabeled DAG)

1. Prepare a "hydrophobic solvent system" and dump it in TLC chamber, cover with lid, and let it sit for a minimum of 1 h before using it.
2. Spot 30 µL of each lower, organic phase sample (from **Subheading 3.1.3.2.**, last step) onto a TLC plate; repeat in rounds until each sample is spotted.
3. Spot 5 µL of each DAG standard onto each plate.
4. Place the plate in the TLC chamber and run it for about 40 min (*see* **Note 9**).
5. Remove plate from the chamber and let it dry.
6. Place plate in iodine chamber to expose standards.
7. Mark standards (R_f of C18:0/C20:4-DAG in this solvent system is ~0.5) and draw lines 1 cm above top standard and 1 cm below standard.
8. Scrape each lane, place scraped silica into scintillation vial containing 4 mL scintillation cocktail and count samples by scintillation spectrometry (*see* **Note 10**).

3.1.4. PLD In Vitro Microassay

PLD catalyzes the hydrolysis of membrane phospholipids (such as PC) releasing phosphatidic acid (PA) and a polar head group. In addition to this, in the presence of a primary alcohol (usually ethanol or 1-butanol), PLD can generate a phosphatidylalcohol [phosphatidylethanol (PEt) or phosphatidylbutanol (PBut)] end-product by what is termed a "transphosphatidylation" reaction. This reaction is unique to PLD and, as a result, is a commonly used method for measuring PLD activity without the possibility of contamination from other phospholipase activities (chiefly the PLC/DAG kinase system that can also generate PA). For the description of the measurement of PLD in this chapter, the substrates or the reaction are: a) a special, short-chain PC (PC8) and b) radiolabeled butanol. The reaction product is phosphatidyl-butanol (PBut) that is separated by TLC in a "polar" solvent. This method was originally described in *(27)*.

3.1.4.1. Preparation of Liposomes

1. Label a small unsiliconized glass test tube "liposomes"; rinse with ddH_2O and dry.
2. Add 13.5 µL (final concentration 24 m*M*) of PC8 per condition to the tube.
3. Dry PC8 lipid under Nitrogen.
4. Once dry, add 22.2 µL of 5 m*M* HEPES per condition to the tube.
5. Vortex vigorously.
6. Let sit for one hour at room temperature.
7. Sonicate for 30 seconds in a bath sonicator; keep liposomes on ice until needed.

3.1.4.2. Preparation of the Assay Mix

1. To a 2-mL Eppendorf tube labeled "assay mix," add the following per condition: 30 µL of 200 m*M* HEPES; 7.89 µL of [^{3}H]butanol and 12.1 µL of ddH_2O.

3.1.4.3. Reaction and Lipid Separation

At this point the enzyme (in cell sonicates) is ready to be mixed with the radiolabeled substrate in the optimal conditions of cofactors, ionic strength, pH, etc.

1. To a 1.5-mL polypropylene tube, add 20 µL of "liposomes" (*see* **Subheading 3.1.4.1.**), 50 µL of "assay mix" (*see* **Subheading 3.1.4.2.**), and 50 µL of "cell sonicates" (*see* **Subheading 3.1.1.**).
2. Include positive controls (cabbage PLD pure enzyme) and negative controls (PLD buffer instead of cell sonicates).
3. Incubate 30 min at 30ºC in a slowly shaking incubator, agitate lightly during the hour.
4. Stop the reaction by adding 3 mL of ice-cold chloroform/methanol (1:2, vol/vol) and 0.7 mL of 1% perchloric acid.
5. Vortex for 1 min and let sit for 10 min.
6. Add 1 mL of chloroform and 1 mL of 1% perchloric acid.
7. Vortex for 1 minute, centrifuge and aspirate upper phase.
8. Wash lower phase with 2 mL of 1% perchloric acid.
9. Vortex, centrifuge, and aspirate upper phase.
10. Repeat wash if necessary.
11. Collect *lower phase* for TLC (should be approx 1 mL).

3.1.4.4. Isolation of Reaction Product (Radiolabeled PBut)

1. Dip a TLC plate in 1.3% potassium oxalate for 1 min.
2. Let dry at room temperature.
3. Heat plate at 115ºC for 1 h; let it cool down.
4. Dry samples under nitrogen.
5. Add 25 µL of chloroform/methanol (95:5, v/v) to dried down sample and then immediately spot 25 µL on the TLC plate—repeat until all samples are spotted.
6. Spot 20 µL of PBut standard on each plate.

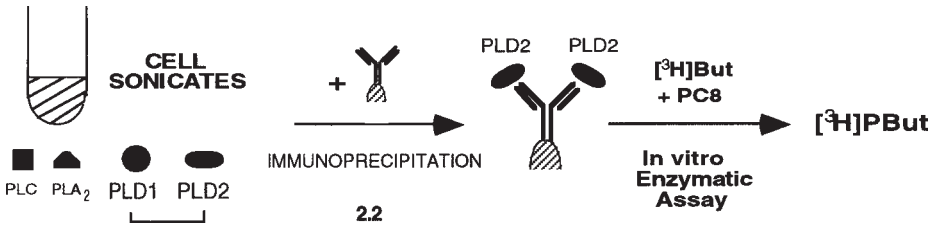

Fig. 3. Immunoprecipitation and immunocomplex enzyme assays. A specific antibody (linked to agarose beads) effectively isolates a particular phospholipase isoform (shown here PLD2) from a cell mix. The activity of the immunoprecipitated enzyme can be assayed then in vitro.

7. Place the plate in the TLC chamber containing the "PLD solvent system" (run takes approx 55 min).
8. Remove plate and let dry.
9. Place plate in iodine chamber to visualize authentic standards.
10. Scrape 0.5 cm above and 2 cm below PBut (R_f in this solvent system is ~ 0.45) (*see* **Note 11**).
11. Count radioactivity.

3.2. Immunoprecipitation and Immunocomplex Enzyme Assays

The immunocomplex assay is an extra step that can be added prior to the in vitro microassays alluded above (*see* **Subheadings 3.1.2–3.1.4.**). This extra step relies on the inclusion of a specific antibody that will ensure that a particular isoform out of a collection of enzymes with similar reaction activities will be measured (**Fig. 3**).

In this chapter, a specific example is given for PLD2 isoform as we have described previously ***(28,29)***. The in vitro microassay described in **Subheading 3.1.4.** will measure *total* PLD in a sample, while immunoprecipitation with PLD2 antibody, as described below, will target that particular activity. This approach can be extrapolated to the study of PLA_2 or PLC isoforms, just by using the appropriate antibody.

3.2.1. Preparation of Antibody Conjugates

With this step the primary antibody will be bound to the secondary antibody (linked to agarose beads) forming the tertiary complex: [1ry/2ry/agarose] or "antibody conjugate." The following amounts are given for one experimental condition. For the experiment, multiply the amounts by the number of conditions and plan for one extra condition for negative controls, i.e., all reagents except the primary antibody. Note that the protocol given below is for PLD2, but it can be easily extrapolated to analyze the PLD1 isoform, or, alternatively, to

analyze particular isoforms of the other phospholipases (PLA_2 and PLC) simply by using the appropriate specific antibody against the isoform intended to be studied. All steps below must be performed at 4ºC.

1. Take 1.5 mL screw cap Eppendorf tube labeled "Ab conjugate" and place on ice; add 40 µL antirabbit beads slurry.
2. Add 100 µL LB to the tube, vortex gently and spin down at 14,000*g* for 15 s and discard supernatant (*see* **Note 12**).
3. Add 40 µL of LB and 60 µL LB/BSA to the beads and vortex gently.
4. Add 1–3 µg of primary antibody (antirabbit PLD2); vortex again.
5. Place the tube in a orbital shaker immediately to rotate upside down overnight at 4ºC (a cold box is best suited for this).

3.2.2. Formation of Immunocomplexes

With this step the tertiary complex: [1ry/2ry/agarose] or "antibody conjugate" (*see* **Subheading 3.2.1.**) will be mixed with the cell sonicates. Here, the primary antibody will bind to its antigen (i.e., PLD protein) forming the immunocomplexes. These can be used in the in vitro microassays for measuring phospholipase activity as indicated earlier.

1. Pellet down live cells (whose phospholipase activity is to be measured) and subject them to lysis. For this, resuspend pellet in 400 µL of LB in an eppendorf tube labeled "lysates." Incubate on ice for 20 min, with one gentle vortexing at 10 min and another 2 or 3 min before the end (*see* **Note 13**).
2. Spin down at 14,000*g* for 1 min, decant and resuspend the pellet in 400 µL LB (*see* **Note 14**).
3. Take the overnight "antibody conjugate" out and spin down at 14,000*g* at 4ºC; carefully remove supernatant, add 100 µL of fresh LB and mix gently (*see* **Note 15**).
4. Add the whole 100 µL of "antibody conjugate" in fresh LB to the "cell lysates" kept on ice per each condition. For negative controls, use just LB instead of cell lysates.
5. Cap tubes, lightly vortex and place in a shaker to rotate at 4ºC for 3 h.
6. Spin down at 4ºC for 15 s at 14,000*g* after the incubation time is up.
7. Dump out supernatant and wash once with 500 µL of prechilled LiCl wash solution and once with 500 µL of prechilled NaCl wash solution.
8. Remove as much of remaining liquid from all tubes without disturbing the pellet and resuspend in 110 µL of LB (*see* **Note 12**). These are the "immunocomplexes" that contain the isoform of the enzyme of interest (PLD_2) in a highly enriched form. They can be split into two 50 µL tubes for a PLD in vitro assay in duplicate as indicated above (*see* **Subheading 3.1.4.3.**, first point). The 50 µL "immunocomplexes" are to be added in lieu of the "cell sonicates" to a 1.5-mL polypropylene tube, along with 20 µL of "liposomes" and 50 µL of "assay mix." Follow the rest of the protocol for the measurement of activity (agarose beads do not interfere with the enzymatic reaction, only make sure that they do not settle down during the incubations).

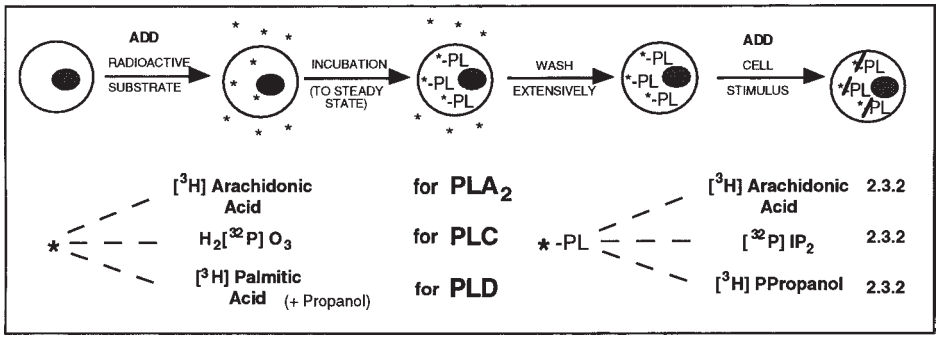

Fig. 4. Intact-cell or in vivo assays. Cultured or suspended cells are incubated with a radioactive precursor: either arachidonic acid, inorganic phosphate or myristic acid, for the measurement of PLA_2, PLCβ, or PLD, respectively. Once a steady state has been reached and the cellular lipids become labeled, cells are washed and stimulated with the agonist under study. This can cause the breakdown of the radiolabeled phospholipids and the release of either arachidonic acid, PIP_2 or phosphatidylpropanol, that are isolated and purified. (*, radiolabeled substrate; PL, phospholipid).

3.3. Intact-Cell (In Vivo) Assays

We now describe the intact-cell or in vivo assays (*see* **Subheadings 3.3.1.** for PLA_2 (*c*PLA_2); **Subheading 3.3.2.** for PLC (PLCβ); and **Subheading 3.3.3.** for PLD) in which living cells are labeled with a radioactive substrate until steady state is reached. Then the intact cells are challenged with an appropriate stimulus and the product of the reaction is isolated from the cells (**Fig. 4**). The advantages of assaying phospholipases in intact cells are: a) One can assay receptor-mediated activation. It is sometimes difficult to maintain the coupling in broken-cell preparations (enriched membrane fractions, whole lysates or sonicates); b) if the conditions of the in vitro assays are set at optimal (as described previously), they certainly will detect all activity there is there, but it might not necessarily reflect what is happening in the intact cell. The phospholipase under study might be down-regulated in the intact cell, but not in the in vitro assay.

3.3.1. Measurement of cPLA$_2$ in Intact Cells or In Vivo

The activity of cytosolic phospholipase A_2 ($cPLA_2$) in vivo is determined by measuring the amount of arachidonic acid released from cells prelabeled with [^{3}H]arachidonic acid.

3.3.1.1. Cell Labeling with [^{3}H]AA

1. Sample [^{3}H]arachidonic acid into a round bottom flask at the final concentration of 1–3 µCi/mL; evaporate the ethanol under a Nitrogen stream ***(30)***.

2. Add 1 mL per experimental condition of a cell suspension (at a 1–5 × 10^7 cells/mL density).
3. Incubated at 37ºC for an hour or until steady state is reached (*see* **Note 16**).
4. Pellet cells (2000*g*, 5 min) and wash twice with Hanks' buffer to eliminate non-incorporated [^{3}H]AA (*see* **Note 17**).
5. Resuspend cells in Hanks' buffer at a 1 × 10^7 cells/mL density. Place cell suspension in a 50-mL conical tube in a reciprocating water bath at 37ºC. Initiate the reaction by the addition of the stimulus (e.g., PMA, 50 ng/mL) to the suspended cells ***(31)***.

3.3.1.2. Measurement of Total AA to the Incubation Media

This method measures the radioactivity in both AA and any labeled metabolites of AA. The main advantage of this method is its simplicity. The disadvantage is that it measures only the radioactivity that is released into the suspending media. Accordingly, it underestimates the amount of AA released.

1. Start with a steady state-labeled, agonist-stimulated cell suspension from **Subheading 3.3.1.1.**
2. Remove 1-mL aliquots at a preset time (e.g., 5–10 min for PMA stimulation); pellet down cells (14,000*g*, 30 s, 4ºC). Keep tubes on ice (repeat this procedure for each time point in a time-course experiment).
3. Transfer 100 µL of the supernatant to scintillation vials and count radioactivity in a β-scintillation counter.

3.3.1.3. Measurement of Cell-Released AA

The advantage of this method over the preceding one is that it measures total AA released. There are two main disadvantages. First, it is time consuming and not simple. Second, it does not account for any possible metabolism of arachidonic acid. This is very minor if the reaction is carried out rapidly ***(32,33)***.

1. Start with a steady state-labeled, agonist-stimulated cell suspension from **Subheading 3.3.1.1.**
2. Pipet down 1 mL aliquots and transfer to glass tubes.
3. Stop the reaction after a preset time by the addition of 100 µL hexane/isopropanol 0.1 N HCl 300:200:4 (v/v/v) (5 vol of stopping solution to 1 vol of suspended cells).
4. For lipid isolation, first vortex mixture and keep overnight at 4ºC ***(32–35)***.
5. The next day, vortex again for 1 min; centrifuge at low speed (2000*g*, 5 min) to separate the two phases.
6. Transfer the *upper phase* ("organic") phase to a new set of clean tubes. Add 2 mL of *n*-hexane to the remaining lower ("aqueous") phase in old tubes; vortex and centrifuge as above. Transfer the upper phase and combine with the previous extraction.
7. Dry the combined organic phase under a Nitrogen stream; resuspend the lipids in a small volume (50–100 µL) of hexane/isopropanol, 3:2 v/v.

8. Spot aliquots (20 µL) on silica gel 60-precoated TLC plates.
9. Add an aliquot (10 µL of a 10 mg/mL solution) of authentic, nonlabeled, AA at the origin with the experimental samples to help detection of AA spots after the plate is developed.
10. Develop plates in the solvent system chloroform/acetone, 96:4 (v/v).
11. Dry plate and expose to I_2 vapors to visualized lipids; circle the spots of authentic AA with a pencil; spray plates lightly with water; scrape the identified silica zones with a razor blade and count for radioactivity.

3.3.2. Measurement of PLCβ in Intact Cells or In Vivo

The activity of PLCβ in vivo is determined either by measuring the amount of IP_3 released or the hydrolysis of PIP_2 from cells prelabeled with radioactive inorganic phosphate ***(32–35)***. The procedure described here measures the latter and the extraction protocol assures the recovery of ~80% of PIP_2 and phosphatidylinositol 4-monophosphate. Differently from the protocols studied before, it makes use of X-ray films to expose radioactivity.

3.3.2.1. Hydrolysis of PIP_2

1. Add a known volume of carrier-free $H_2[^{32}P]O_3$ (final concentration 33 µCi/mL) to a cell suspension (at a 1–5 × 10^7 cells/mL density).
2. Incubate cells at 37ºC for 1 h or until steady state is achieved (*see* **Note 16**).
3. Pellet cells (2000*g*, 5 min) and wash twice with Hanks' buffer to eliminate nonincorporated $H_2[^{32}P]O_3$ (*see* **Note 17**).
4. Resuspend cells in Hanks' buffer at a 1 × 10^7 cells/mL density. Initiate the reaction by the addition of the stimulus of choice to the cell suspension.
5. Extract lipids and apply TLC plates as indicated in **Subheading 3.3.1.3. steps 1–8**.
6. Develop plates in one dimension in chloroform/methanol/20% methylamine (60:36:10 v/v).
7. Dry plates at room temperature and place them in a Kodak exposure cassette with an X-ray film overnight to visualize the radioactive lipids.
8. The following day, develop the film; carefully realign film and plate to equate the exposure position; locate lipids and puncture the film and the silica layer under it with a needle. Next, scrape lipids of interests with a razor blade and count radioactivity (*see* **Note 18**).

3.3.3. Measurement of PLD in Intact Cells or In Vivo

The activity of PLD in vivo is determined by measuring the amount of radiolabeled phosphatidylpropanol (PProp) synthesized in cells prelabeled with [^{3}H]palmitic acid and in the presence of a primary alcohol (1-propanol) ***(36)***. As indicated in **Subheading 3.1.4.** PLD can generate a phosphatidylalcohol (in this case PProp) by the "transphosphatidylation" reaction.

1. Plate out cells in 12-well plates at the density of 1×10^6/per well.
2. Prepare tissue culture media (e.g., RPMI1640) containing 15 µCi/mL [^{3}H]palmitic acid (final concentration).
3. Add 0.8 mL per well and incubate cells for 24 h or until steady state is achieved (*see* **Note 16**).
4. Remove all the media from the wells, one well at a time (as to keep the wells from drying out) and add 0.8 mL of fresh, nonradioactive, EBS media (serum free).
5. Remove the media over the cells and replace with 500 µL of EBS. Preincubate 10 min at 30ºC; change the media to 500 µL of EBS containing the stimulus to be tested (e.g., PMA) plus 1-propanol (1% final concentration).
6. At the end of the incubation, transfer media to a 15-mL tube, leave it on ice.
7. Add 1 mL of methanol to the wells, and put the plate on ice. Centrifuge the 15-mL tube, remove the supernatant. Scrape the cells from the well and transfer to the corresponding 15-mL tube. Add an additional 1 mL of methanol to the well, scrape and transfer to the corresponding tube. Vortex and then let the tube sit on ice as you complete the other extractions.
8. Add 1 mL of chloroform and 0.7 mL of 1% perchloric acid to each tube, vortex for 1 min. Let stand for 10 min at room temperature.
9. Add 1 mL of chloroform, 1 mL of 1% perchloric acid, vortex 1 min, and aspirate the upper phase.
10. Wash lower phase twice with 2 mL of 1% perchloric acid.
11. Remove two 500-µL aliquots from *lower phase* for chromatography, store in freezer. Count a 50-µL aliquot to determine the total amount of labeled phospholipids.
12. Run TLC as indicated in **Subheading 3.1.4.4.** Spot 5 µL of the "standard mix" over each sample and 5 µL on an extra lane by itself; dry plate, visualize lipids with I_2 vapors and scrape the spots that comigrate with PProp ($R_f = 0.5–0.6$), and count.

4. Notes

1. Authentic standards are always kept under N_2 in a sealed tube, wrapped up in aluminum foil to avoid direct light, and placed in a desiccator inside a deep (–70ºC) freezer.
2. The hydrophobic solvent system for TLC separation *must* be prepared fresh for each use.
3. The HEPES, $MgCl_2$, and EGTA solutions are prepared in small plastic bottles and kept at 4ºC for up to 6 mo. The protease/phosphatase inhibitors are prepared from lyophilized samples, immediately aliquoted down and stored at –70ºC protected from light and with a desiccant (in this way they are still effective up to three months). At the time of the experiment, thaw them out and add to the base buffer. Discard aliquots after using, never refreeze.
4. It is crucial that the primary and secondary antibodies are of the same species. For a monoclonal (mouse) primary antibody always use antimouse IgG secondary antibody linked to agarose beads (available from Sigma). For a polyclonal (rabbit) primary antibody always use antirabbit IgG secondary antibody linked to agarose beads (also available from Sigma).

5. Add appropriate cell stimuli (at variable concentration or time of incubation) whose action on the enzyme activity is to be tested. Some phospholipases (like PLA_2) will benefit from the presence of calcium in the media. To this end, preincubate cells with 1.6 *M* $CaCl_2$ at 37ºC for 5 min. Because cells tend to sediment, make sure the tube is being rotated properly in the water bath. To stimulate cells, use 1 µL stimulus stock per 1 mL of cells. In this way the stimulus is diluted 1000 times. Some stimuli might require an intermediate dilution. For example, when using PMA, we usually prepare a 500 µg/mL (in DMSO) stock from which we make a 1:10 dilution in Hanks' buffer right before the experiment. We then add 1 µL per milliliter of cell suspension (final concentration is 50 ng/mL). All intermediate and final dilutions are discarded after use and never refrozen. Whenever adding the stimulus to the cell suspension, hold conical tube in hand as swirling it, to facilitate immediate dispersion; add the stimulus very slowly to the suspension going back and forth several times with the pipette to completely release the contents. Place conical tube on bath.
6. As an alternative to sonication, cell lysis can be performed in "lysis buffer": 12 m*M* Tris-HCl, pH 7.2; 14 m*M* NaCl; 0.75 m*M* EDTA; protease and phosphatase inhibitors as described, and 0.25% Triton X-100. Mix cell pellets with lysis buffer and carry out lysis on ice for 20 min with intermittent (every 3–5 min) vortexing. Spin lysates at 14,000*g* and collect supernatant. We have found ***(27)*** that Triton X-100 is inhibitory for PLD, thus, a different detergent must be used. β-octyl-glucopyranoside, in our hands, is appropriate, as long as it is kept at a final concentration of 0.05%, well below its critical micelle concentration (CMC).
7. The measurement of cytosolic $cPLA_2$ activity in vitro can be determined by measuring the amount of AA released using 1-stearoyl-2-[1-^{14}C]arachidonyl-phosphatidylcholine as a substrate ***(24)***. The substrate is dried under a stream of nitrogen and resuspended in DMSO with vigorous vortex-mixing. A known volume of the substrate (2 µL) (final concentration 15 µ*M*) and 5 µL of 16 m*M* $CaCl_2$ (final concentration 2 m*M*) are added to eppendorf microcentrifuge tubes. The reaction is initiated by adding a known volume (33 µL, 30–40 µg protein) of the cytosolic fraction of cell homogenate. This is obtained by suspending a known number of cells (1–5 × 10^7 cells) in 1.2 mL lysis buffer. The mixture is sonicated and then centrifuged at 150,000*g* for 20 min. The supernatant obtained is used to measure $cPLA_2$ activity. The reaction is carried out for a preset time (5–40 min), and it is stopped by adding a known volume (40 µL) of ice-cold quench solution, comprising 40 µg/mL AA in ethanol containing 2% (vol/vol) acetic acid. A 40-µL of the solution is spotted on LK5DF silica-gel TLC plates and developed in organic phase of ethyl acetate/iso-octane/acetic acid/water (55:75:8:100, by volume). The area corresponding to AA is revealed by brief exposure to iodine vapor, scraped, and the radioactivity is counted.
8. An alternative procedure for separation of the [^{3}H]AA product, is to apply upper phase (from **Subheading 3.1.2.2.**, last step) to a silica gel plate and TLC and develop it in the TLC solvent system hexane:diethylether:formic acid (60:40:2, v/v/v) (R_f is ~0.7). This system allows the quantification of total [^{3}H]PC that should remain in, or very close to, the application spot after running the plate (R_f is ~0–0.05).

9. To run a plate in this "nonpolar" solvent mixture takes approx 40 min, much shorter than other chromatography system, thus it must be watched closely or it will overrun.
10. After plate has run, a zone around (0.5 cm above and below) the application spot (R_f ~0) could also be scraped to analyze total [^{14}C] PC. As PC is broken down by PLC to form PA, the amount of PC should *decrease*.
11. We routinely use a long-chain analog of PC8-PBut because, as far as we can tell, there are not commercially available standards for PBut with the short, dioctanoyl chain (PC8). In the TLC system considered, the R_f for PC8-PBut is located approx 1 cm below the long-chain PBut (and actually close to phosphatidylethanol (PEt) that can be used as an alternative standard).
12. During all steps that require washes during the immunoprecipitation procedure, always pull off supernatant very carefully, leaving a small amount of liquid on the precipitate so as to avoid touching and unsettling the beads. Alternatively, the tube can be decanted, positioning it at a 45º angle with the mouth of the tube against a paper napkin/kimwipe. Never overdo it (it is better to leave some liquid on top of the beads pellet, forming a meniscus), because it is vital that beads remain packed at the bottom of the Eppendorf and are not lost.
13. In order to increase the yield of protein, sometimes it helps to sonicate the lysates briefly (this should always be done on ice): two cycles (on mid-high setting of sonicator), one for 8 s then for 6 s and let settle on ice for 10–20 min.
14. Because imunoprecipitation requires only 200 µL of sample, the rest of the lysates could be stored frozen at −70ºC for future use. We routinely save 20 µL aliquots for protein determination the same day of the experiment.
15. Cut a small amount from the end of the yellow tips and resuspend 1–2 times to ensure that the beads will not clog the end of the tip. Vortex tube of immunocomplex beads often to ensure that beads remain in suspension all the time.
16. This period of incubation varies, but the goal is to achieve steady state. This is accomplished when cell-associated radioactivity does not change with time. The time needed to reach steady state varies among different cells.
17. Hanks buffer is ideal for neutrophils, but any desired buffered solution can be used. For cultured cells, fresh media is fine. The goal of the washes is to remove any nonincorporated labeled AA. Thus, the washing must be thorough. A sample of the supernatant of the last wash can be quickly counted for any residual radioactivity. If necessary, additional washing may be required.
18. To help locate the position of inositol-containing phospholipids, it is useful to label an aliquot of the cells with [^{3}H]inositol instead of $H_2[^{32}P]O_3$ (this can be done at point 4). A sample is then taken for lipid extraction and TLC application along with the experimental samples.

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References

1. Jalink, K., van Corven, E. J., and Moolenaar, W. H. (1990) Lysophosphatidic acid, but not phosphatidic acid, is a potent Ca^{2+}-mobilizing stimulus for fibroblasts. Evidence for an extracellular site of action. *J. Biol. Chem.* **265,** 12,232–12,239.
2. Venable, M. and Obeid, L. M. (1999) Phospholipase D in cellular senescence. *Biochim. Biophys. Acta* **1439,** 291–298.
3. Griffiths, R. J. (1999) Prostaglandins and inflammation, in *Inflammation: Basic Principles and Clinical Correlates,* Chapter 22, 3rd edition, (Gallin, J. I. and Snyderman, R., eds.), Williams & Wilkins, Philadelphia, PA, pp. 349–360.
4. Nishizuka, Y. (1992) Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* **258,** 607–614.
5. Berridge, M. J. (1993) Inositol trisphosphate and calcium signaling. *Nature* **361,** 315–325.
6. Jimenez, B., del Peso, L., Montaner, S., Esteve, P., and Lacal, J. C. (1995) Generation of phosphorylcholine as an essential event in the activation of Raf-1 and MAP-kinases in growth factors-induced mitogenic stimulation. *J. Cell Biochem.* **57,** 141–149.
7. van Dijk, M. C., Hilkmann, H., and van Blitterswijk, W. J. (1997) Platelet-derived growth factor activation of mitogen-activated protein kinase depends on the sequential activation of phosphatidylcholine-specific phospholipase C, protein kinase C-ζ and Raf-1. *Biochem J.* **325,** 303–307.
8. Kiss, Z. and Mukherjee, J. J. (1997) Phosphocholine and sphingosine-1-phosphate synergistically stimulate DNA synthesis by a MAP kinase-dependent mechanism. *FEBS Lett.* **412,** 197–200.
9. Moolenaar, W. H., Kruijer, W., Tilly, B. C., Verlaan, I., Bierman, A. J., and de Laat, S. W. (1986) Growth factor-like action of phosphatidic acid. *Nature* **323,** 171–173.
10. Kaszkin, M., Richards, J., and Kinzel, V. (1996) Phosphatidic acid mobilized by phospholipase D is involved in the phorbol 12-myristate 13-acetate-induced G_2 delay of A431 cells. *Biochem. J.* **314,** 129–138.
11. Carnero, A. J. and Lacal, J. C. (1993) Phospholipase-induced maturation of *Xenopus laevis* oocytes: mitogenic activity of generated metabolites. *J. Cell. Biochem.* **52,** 440–448.
12. Kaszkin, M. (1992) Proposed role of phosphatidic acid in the extracellular control of the transition from G_2 phase to mitosis exerted by epidermal growth factor in A431 cells. *Cancer Res.* **52,** 5627–5634.
13. Carnero, A., Cuadrado, A., del Peso, L., and Lacal, J. C. (1994) Activation of type D phospholipase by serum stimulation and Ras-induced transformation in NIH3T3 cells. *Oncogene* **9,** 1387–1395.
14. Kiss, Z. and Crilly, K. S. (1996) Ethanolamine analogues stimulate DNA synthesis by a mechanism not involving phosphatidylethanolamine synthesis. *FEBS Lett.* **381,** 67–70.
15. Andrews, B., Bond, K., Lehman, J. A., Horn, J. M., Dugan, A., and Gomez-Cambronero, J. (2000) Direct inhibition of *in vitro* PLD activity by 4-(2-aminoethyl)-benzenesulfonyl fluoride. *Biochem. Biophys. Res. Com.* **273,** 302–311.

16. Lin, L. L., Lin, A. Y., and Knopf, J. L. (1992) Cytosolic phospholipase A_2 is coupled to hormonally regulated release of arachidonic acid. *Proc. Natl. Acad. Sci. USA* **89,** 6147–6151.
17. Leslie, C. C. (1997) Properties and regulation of cytosolic phospholipase A_2. *J. Biol. Chem.* **272,** 6709–16712.
18. Piomelli, D. (1993) Arachidonic acid in cell signaling. *Current Opinion Cell Biol.* **5,** 274–280.
19. Hajjar, J.-J., Rezaul, K., and Sha'afi, R. I. (2000) Cytosolic phospholipase A_2 and arachidonic acid release. *Current Top. Biochem. Res.* **3,** 1–25.
20. Borsch-Haubold, A. G., Kramer, R. M., and Watson, S. P. (1995) Cytosolic phospholipase A_2 is phosphorylated in collagen- and thrombin-stimulated human platelets independent protein kinase C and mitogen-activated protein kinase. *J. Biol. Chem.* **270,** 25,885–25,892.
21. Kramer, R. M., Roberts, E. F., Hyslop, P. A., Utterback, B. G., Hui, K. Y., and Jakubowski, J. A. (1995) Differential activation of cytosolic phospholipase A_2 ($cPLA_2$) by thrombin and thrombin receptor agonist peptide in human platelets. *J. Biol. Chem.* **270,** 14,816–14,823.
22. Waterman, W. H., Molski, T. F. P., Huang, C.-K., Adams, J. L., and Sha'afi, R. I. (1996) TNF-α-induced phosphorylation and activation of cytosolic phospholipase A_2 are abrogated by an inhibitor of the p38 MAP kinase cascade in human neutrophils. *Biochem. J.* **319,** 17–20.
23. Muthalif, M. M., Hefner, Y., Harper, J., Parmentier, J. H., Achersold, R., Gelb, M. H., and Malik, K. U. (2001) Functional interaction of calcium/calmodulin-dependent protein kinase II and cytosolic phospholipase A_2. *J. Biol. Chem.* **298,** 272–278.
24. Nahas, N., Waterman, W. H., and Sha'afi, R. I. (1996) Granulocyte-macrophage colony- stimulating factor (GM-CSF) promotes phosphorylation and an increase in the activity of cytosolic phospholipase A_2 in human neutrophils. *Biochem. J.* **313,** 503–508.
25. Van Den Bosch, H., De Jong, J. G. N., and Aarsman, A. J. (1991) Phospholipase A_2 from rat liver mitochondria. *Methods Enzymol.* **197,** 365–376.
26. Waite, M. and Smith, D. (1992) Phosphatidylinositol hydrolysis by and phospholipase A_2 and C activities in human peripheral blood neutrophils. *J. Leukocyte Biol.* **52,** 670–678.
27. Davis, L. L., Maglio, J. J., and Horwitz, J. (1998) Phospholipase D hydrolyzes short-chain analogs of phosphatidylcholine in the absence of detergent. *Lipids* **33,** 223–227.
28. Horn, J. M., Lehman, J. A., Alter, G., Horwitz, J., and Gomez-Cambronero, J. (2001) Presence of a phospholipase D (PLD) distinct from PLD1 or PLD2 in human neutrophils: immunobiochemical characterization and initial purification. *Biochim. Biophys. Acta* **1530,** 97–110.
29. Blum, J. J., Lehman, J. A., Horn, J. M., and Gomez-Cambronero, J. (2001) Phospholipase D (PLD) is present in *Leishmania donovani* and its activity increases in response to acute osmotic stress. *J. Eukaryotic Mic.* **48,** 102–110.
30. Rhe, S. G. and Bae, Y. S. (1997) Regulation of phosphoinositide-specific phospholipase C. *J. Biol. Chem.* **272,** 15,045–15,048.

31. Syrbu, S. I., Waterman, W. H., Molski, T. F. P., Nagarkatti, D., Hajjar, J.-J., and Sha'afi, R. I. (1999) Phosphorylation of cytosolic phospholipase A_2 and the release of arachidonic acid in human neutrophils. *J. Immunol.* **162,** 2334–2340.
32. Volpi, M., Yassin, R., Tao, W., Molski, T. F. P., Naccache, P. H., and Sha'afi, R. I. (1984) Leukotriene mobilizes calcium without the breakdown of polyphosphoinositides and the production of phosphatidic acid in rabbit neutrophils. *Proc. Natl. Acad. Sci. USA* **81,** 5966–5969.
33. Volpi, M., Yassin, R., Naccache, P. H., and Sha'afi, R. I. (1983) Chemotactic factor causes rapid decrease in phosphatidylinositol 4,5-bisphosphate and phosphatidyl inositol 4 monophosphate in rabbit neutrophils. *Biochem. Biophys. Res. Com.* **112,** 957–964.
34. Hara, A. and Radin, N. (1978) Lipid extraction of tissues with a low-toxicity solvent. *Anal. Biochem.* **90,** 420–426.
35. Bell, M. E., Peterson, R. G., and Eichberg, J. (1982) Metabolism of phospholipids in peripheral nerve from rats with chronic streptozotocin-induced diabetes: Increased turnover of phosphatidylinositol-4,5-bisphosphate. *J. Neurochem.* **39,** 192–200.
36. Levine, G., Maglio, J., and Horwitz, J. (2000) Differential effects of long-term ethanol on signal transduction in PC12 cells. *Alcoholism: Exp. Clinical Res.* **24,** 93–101.

14

Detection of the Content and Activity of the Transcription Factor AP-1 in a Multistage Skin Carcinogenesis Model

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Abstract

Investigation of transcription factor activity in animal tissue during the early stage of cancer development can be difficult because of a low number of affected cells in a background of a large number of normal cells. We have used a well-established multistage skin carcinogenesis model to study the effect of manganese superoxide dismutase on the activity of AP-1 during an early stage of mouse skin carcinogenesis. The DNA binding activity of AP-1 proteins to a concensus DNA regulatory binding element known as TRE (TPA Responsive Element) is used as an in vitro assay for AP-1 activity in the nucleus of skin cells. This activity is detected by electrophoretic mobility shift assay (EMSA) which is based on the ability of specific proteins to bind and retard the migration of radioactive labeled oligonucleotides on a native gel. The presence of a specific protein in the complex can be identified by adding to EMSA an antibody specific for that protein, which will result in supershift complexes on the gel. The presence of the proteins in question can be further verified by standard Western analysis of the nuclear proteins.

Key Words: Skin; transcription factor; oxidative stress; cancer; MnSOD; EMSA.

1. Introduction

Genetically modified animals, in which the skin serves as the target for studying carcinogenesis, have been particularly valuable in terms of generating mechanistic insights. We have used transgenic mice overexpressing the primary antioxidant

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enzyme Manganese superoxide dismutase (MnSOD) to investigate the mechanisms by which alteration of cellular redox status may modify the carcinogenesis process. MnSOD is a nuclear-encoded primary antioxidant enzyme localized in mitochondria ***(1)***. The known function of MnSOD is to catalyze the dismutation of superoxide radicals, which, if not properly removed, can participate in the generation of highly toxic reactive oxygen and nitrogen species (ROS and RNS), including hydroxyl radical and peroxynitrite anion ***(2,3)***. Several lines of study suggested the possibility that MnSOD may function as a new type of tumor suppressor gene ***(4–6)***. Previous investigations from this and other laboratories have demonstrated that MnSOD may suppress cancer by modulation of redox-sensitive signal mediators leading to alter expression and activity of various transcription factors including the transcription factor activator protein -1 (AP-1) ***(7–10)***.

Investigation of transcription factor activity in animal tissue during the early stage of cancer development can be difficult because of a low number of affected cells in a background of a large number of normal cells. We have used a well-established multistage skin carcinogenesis model developed by Fischer et al. ***(11)*** to study the effect of MnSOD on the activity of AP-1 during an early stage of mouse skin carcinogenesis. In this model, a tumor is initiated by applying a subthreshold dose of 7, 12-dimethylbenz(a)anthracene (DMBA) followed by daily exposure to 12-*O*-tetradecanoylphorbol-13-acetate (TPA). In this protocol, initiation is considered to result in a population of initiated cells that remain dormant until stimulated to expand clonally upon treatment with the promoter, which leads to the formation of benign well-differentiated papillomas. A portion of these papillomas will progress to squamous carcinomas and eventually to invasive undifferentiated carcinomas ***(12)***.

One of the most thoroughly studied classes of tumor promoters, TPA is known to produce reactive oxygen species (ROS) with resultant oxidative damage to macromolecules, leading to changes in cellular redox status ***(13,14)***. It is now well recognized that superoxide radicals, a major intracellular ROS, are not just a toxic byproduct of molecular oxygen, but are also a signaling molecule. It has been shown that ROS can modulate the activity of protein kinases which, in turn, phosphorylates a wide range of cellular proteins ***(15)***. The structure of this group of proteins contains a tandem repeat of cysteine-rich amino acids in their regulatory domain ***(16)***. These amino acids are the target for oxidation. It is interesting to note that although excessive oxidation inactivates protein kinase activity, mild oxidation selectively modifies the regulatory domain of protein kinases causing persistent activation of kinases ***(15,17)***. Activation of protein kinase activity has been widely demonstrated in mouse skin carcinogenesis promoted with TPA ***(10,11,18–20)***. Because the activities of AP-1 components are generally modulated by phosphorylation ***(21)***, the tumor-promoter activity

of TPA may in part be caused by its ability to generate mild oxidative stress and activation of protein kinases leading to increased phosphorylation of Fos and Jun proteins. Because Fos and Jun form a hetrodimeric complex that interacts with the AP-1 binding site and transcription activation by Fos and Jun is a result of the cooperative action of these proteins ***(18)***, in the following sections we describe an approach to determine the activity of AP-1 and its associated components in an early-stage skin carcinogenesis. The DNA-binding activity of AP-1 proteins to a concensus DNA regulatory binding element known as TRE (TPA Responsive Element) is used as an in vitro assay for AP-1 activity in the nucleus of skin cells. This activity is detected by electrophoretic mobility shift assay (EMSA), which is based on the ability of specific proteins to bind and retard the migration of radioactive-labeled oligonucleotides on a native gel. The presence of a specific protein in the complex can be identified by adding to EMSA an antibody specific for that protein, which will result in supershift complexes on the gel. The presence of the proteins in question can be further verified by standard Western analysis of the nuclear proteins.

2. Materials

2.1. Animals

Transgenic mice expressing the human *MnSOD* gene were generated and characterized in our laboratory as previously described ***(22,23)***. Mice used for producing transgenic mice were the F1 hybrid of C57BL/6 mice crossed with C3H mice (B6C3). For the skin carcinogenesis experiments, female transgenic mice that exhibited a high level of *MnSOD* activity (TgH) and their female nontransgenic littermates (nTg) were used.

2.2. Nuclear Fraction Isolation

1. Buffer A: 10 m*M* HEPES-KOH, pH 7.9 (autoclaved), 1.5 m*M* $MgCl_2$, 10 m*M* KCl, 0.2 m*M* phenylmethylsulfonyl fluoride (PMSF), 5 µ*M* of dithiothreitol (DTT), and 5 µg/mL of protease inhibitors consisiting of pepstatin, leupeptin, and aprotinin.
2. 10% NP-40 (1 mL NP-40 diluted in 9 mL autoclaved distilled H_2O).
3. Buffer B: 20 m*M* HEPES-KOH, pH 7.9 (autoclaved), 1.5 m*M* $MgCl_2$, 420 m*M* NaCl, 35% glycerol, 0.2 m*M* PMSF, 5 µ*M* of DTT, 0.2 m*M* ethylenediamine tetraacetic acid (EDTA), pH 8.0, and 5 µg/mL of protease inhibitors consisting of pepstatin, leupeptin, and aprotinin.

2.3. Electrophoretic Mobility Shift Assays

1. The AP-1 double-stranded oligonucleotides: 5'-CGCTTGATGAGTCAGCCGGA A-3' 5'-TTCCGGCTGACTCATCAAGCG3'
2. 5X binding buffer: 50 m*M* Tris-HCl, pH 7.4, 20% glycerol, 5 m*M* $MgCl_2$, 2.5 m*M* EDTA, 5 m*M* DTT, and 0.25 mg/mL poly dI-dC.

3. Glass plate (15 × 17-cm) for casting gels.
4. 1.5-mm spaces and 20-tooth combs.
5. 30% acrylamide/*bis*-acrylamide (29:0.8) solution.
6. 5X Tris-borate-EDTA (TBE) buffer, pH 8.3.
7. 10% ammonium persulfate, freshly made.
8. N,N,N',N'-tetramethylenediamine (TEMED).
9. 10X sample loading buffer: 250 m*M* Tris-HCl, pH 7.5, 40% glycol, 0.2% bromophenol blue.
10. Gel apparatus and power supply.

2.4. SDS Gel Electrophoresis and Western Blotting

1. 10X electrode buffer: 30.3 g Tris-HCl, 144 g glycine, 10 g SDS, distilled H_2O to 1 L, pH 8.3.
2. 5X separation buffer: Tris-HCl 56.76 g, SDS 1.25 g, distilled H_2O to 250 mL, pH 8.8.
3. 5X stacking buffer: Tris-HCl 37.85 g, SDS 2.5 g, distilled H_2O to 500 mL, pH 6.8.
4. 2X sample loading buffer: Tris-HCl 0.76 g, SDS 2 g, glycerol 10 mL, 2-mercaptoethanol 5 mL, distilled H_2O to 50 mL, pH 6.8.
5. Blotto blocking buffer: 5% milk, 10 mm Tris-HCl (pH 8.0), 150 m*M* NaCl, and 0.05% Tween-20.
6. TBST: 10 m*M* Tris-HCl with 150 m*M* NaCl and 0.05% Tween-20.
7. TBS: 10 m*M* Tris-HCl with 150 m*M* NaCl.
8. Enhanced chemiluminescence detection system (ECL, Amersham Pharmacia Biotech, Piscataway, NJ).

2.5. Antibodies

Rabbit polyclonal antibodies against *c*-Jun, Jun B, Jun D, *c*-Fos, Fra-1 (Santa Cruz Biotechnology).

3. Methods

3.1. Carcinogens Treatment

1. With a rodent electric shaver, remove hair from the backs of mice (*see* **Note 1**) 2 d prior to application of vehicle or carcinogen.
2. Apply 200 µL of 20 nmol DMBA (Sigma, St. Louis, MO) dissolved in dimethysulfoxide (DMSO) to the back of each mouse (*see* **Note 2**).
3. Two weeks after DMBA application, apply daily (Monday to Friday) to the same area, for 14 wk, 200 µL containing 4 µg of TPA, also dissolved in DMSO.

3.2. Preparation of Skin Cells from Mice

1. Euthanatize mice with an overdose of pentobarbital (150 mg/kg/ip).
2. Using forceps and sharp scissors, remove the entire painted area of the skin (approx 1-in square).
3. Place the skin with the epidermis side up on an ice-cold glass plate.

4. Trim dermal tissues using sterile scissors and forceps.
5. Strip off skin cells (*see* **Note 3**) using an autoclaved glass slide.

3.3. Preparation of Nuclear Extract from Skin Cells (see Note 4)

1. Suspend the stripped skin cells in a 1.5-mL mircrocentrifuge tube with 400 µL buffer A.
2. Gently vortex the sample using a vertex mixer at low speed.
3. Centrifuge the sample for 10 s in a low-speed table-top centrifuge.
4. Transfer the supernatant to a new tube.
5. Resuspend the pellet in 400 µL buffer A and gently homogenize the sample (15 times up and down) with a 10-mL Wheaton homogenizer, transfer to a new 1.5 mL microcentrifuge tube, and centrifuge the homogenate for 10 s in a low-speed table-top centrifuge.
6. Add the supernatant to the first supernatant, collected in **step 4**.
7. Incubate the combined supernatant on ice for 30 min, then add 25 µL of 10% NP-40, and vortex vigorously for 25 s.
8. Centrifuge the lysate at 17,500*g* for 1 min and remove the supernatant.
9. Wash the pellet once with buffer A, then dissolve the pellet in 120 µL buffer B.
10. Incubate the sample on ice for 30 min followed by centrifugation at 13,000*g* for 5 min, and then collect the supernatant, which is identified as *nuclear extract.*
11. Determine the protein concentration of the nuclear extract, aliquot, and store at –80ºC.

3.4. Electrophoretic Mobility Shift Assays (EMSAs)

1. Prepare the gel mix by combining 32.1 mL distilled H_2O, 5 mL 5X TBE, 10 mL 30% acrylamide, 2.5 mL 50% glycerol, 375 µL 10% APS, 25 µL TEMED (50 mL total volume). Allow the gel to polymerize for at least 2 h. Before loading the sample, pre-run the polymerized gel in 0.5X TBE at 100 V at room temperature for 30 min.
2. Label the probe by combining 18.5 µL distilled H_2O, 3 µL T4 Kinase 10 X Buffer, 2.5 µL AP-1 double-stranded oligonucleotides, 4 µL γ-p^{32}-ATP (3000Ci/mmole at 10 mCi/mL), and 2 µL T4 polynucleotide kinase (30 µL total volume). Incubate the mixture for 1 h at 37ºC; purify the probe by separating the labeled oligonucleotides from free isotopes on a 20% native polyacrylamide gel; expose the gel briefly to an X-ray film, develop the film; excise the band corresponding to the double-stranded oligonucleotides from the gel with the aid of the autoradiograph of the gel; break the excised gels into tiny pieces and incubate in 400 µL TE buffer in a 1.5-mL microcentrifuge tube at 37ºC overnight; centrifuge briefly to pellet the gels, transfer the supernatant to a new tube; count the radioactivity of the labeled probe in a scintillation counter and store at –80ºC.
3. Analyze AP-1-DNA binding activity in *nuclear extracts* by incubation of 25-µL reaction solution containing 6 µg of *nuclear extract*, 5 µL of 5X binding buffer, and 50,000 cpm labeled AP-1 oligo concensus for 20 min at room temperature.

After incubation, add 3 µL of 10X loading buffer and separate the samples on a 6% native polyacrylamide gel for 3 to 4 h (*see* **Note 5**). Detect the DNA-protein complexes by exposing the gels to Kodak film at –80ºC.

4. For supershift assays, pre-incubate the *nuclear extract* with 5 µg of the respective antibody to each member of the AP-1 family (anti-*c*-Jun, Jun B, Jun D, *c*-Fos, Fra-1) for 1 h at room temperature, and then perform EMSA as described earlier (*see* **Note 6**).

3.5. Western Blot Analysis

1. Prepare the separation gel by combining 16.1 mL distilled H_2O, 7 mL 5X separation buffer, 11.7 mL 30% acrylamide, 150 µL 10% APS, 25 µL TEMED (35 mL total volume). Allow the gel to polymerize for 1 h.
2. Prepare the stacking gel by combining 7 mL distilled H_2O, 2 mL 5X stacking buffer, 1 mL 30% acrylamide, 50 µL 10% APS, 10 µL TEMED (10 mL final). Allow the gel to polymerize for 1 h.
3. To detect Jun D and *c*-Jun, load 30 µg of the *nuclear extract* on a 10% SDS-PAGE gel. After transferring, stain the gels with Ponceau to assess transfer of protein onto the nitrocellulose membrane and block potential nonspecific binding by incubation with Blotto for 2 h at room temperature and then incubate the membranes with a rabbit polyclonal antibody against Jun D (Santa Cruz Biotechnology) at a 1:1000 dilution. Before adding the secondary antibody, wash the membrane twice with TBST (10 m*M* Tris-HCl with 150 m*M* NaCl and 0.05% Tween 20), and then incubate the membrane with horseradish peroxidase conjugated secondary antibodies (Santa Cruz Biotechnology) at a 1:4000 dilution for 1–2 h in room temperature or overnight in a cold room with shaking. The final washing steps include three times (5 min each) with TBST and two times (5 min each) with TBS (10 m*M* Tris-HCl with 150 m*M* NaCl). The antibody bands were visualized by the enhanced chemiluminescence detection system as described by the supplier (ECL, Amersham Pharmacia Biotech, Piscataway, NJ). The membrane can be stripped and reprobed with an anti-*c*-Jun antibody (Santa Cruz Biotechnology) at a 1:1000 dilution.

4. Notes

1. Female mice are used because they are slightly more sensitive to developing papillomas with this protocol. Younger mice are also more sensitive, and thus we generally use mice 6–8 wk old.
2. Only mice in the hair cycle resting stage are selected for subsequent treatments.
3. We collect mostly the cells in the basal and suprabasal layers of the mouse skin epidermis.
4. To obtain as many intact skin cells possible, without cell debris, the cells were initially collected from the first supernatant before homogenization. The resulting pellet is then gently homogenized to release additional skin cells. The samples should be kept on ice during homogenization. If a motorized homogenizer is used; it should be set to the lowest position.

5. The front dye runs to about two-thirds of the whole gel. To make the binding band sharper, the electrophoresis can be stopped when the front dye reaches the middle of the gel.
6. For supershift, the gel should be run longer to allow the antibody-protein-DNA complex to enter the gels. We usually stop the electrophoresis when the Bromophenal blue, which is the front dye, just runs off the gel.

References

1. Weisiger, R. A. and Fridovich, I. (1973) Superoxide dismutase: Organelle specificity. *J. Biol. Chem.* **248,** 4793–4796.
2. Halliwell, B., Zhao, K., and Whiteman, M. (1999) Nitric oxide and peroxynitrite. The ugly, the uglier and the not so good: a personal view of recent controversies. *Free Radic. Res.* **31(6),** 651–669.
3. McCord, J. M. and Fridovich, I. (1988) Superoxide dismutase: the first twenty years (1968–1988). *Free Radic. Biol. Med.* **5(5–6),** 363–369.
4. Oberley, L. W. and Buettner, G. R. (1979) Role of superoxide dismutase in cancer: a review. *Cancer Res.* **39,** 1141–1149.
5. Sun, Y. (1990) Free radicals, antioxidant enzymes, and carcinogenesis. *Free Radic. Biol. Med.* **8,** 583–599.
6. St. Clair, D. K. (1996) Tumor suppression by manganese superoxide dismutase: A novel function of MnSOD? A commentary. *Oncol. Rep.* **3,** 429–432.
7. Kiningham, K. K. and St. Clair, D. K. (1997) Overexpression of manganese superoxide dismutase selectively modulates the activity of Jun-associated transcription factors in fibrosarcoma cells. *Cancer Res.* **57,** 5265–5271.
8. Manna, S. K., Zhang, H. J., Yan, T., Oberley, L. W., and Agrawal, B. B. (1998) Overexpression of manganese superoxide dismutase suppresses tumor necrosis factor-induced apoptosis and activation of nuclear transcription factor κB and activated protein-1. *J. Biol. Chem.* **273,** 13,245–13,254.
9. Zhao, Y., Kiningham, K. K., Lin, S.-M., and St. Clair, D. K. (2001) Overexpression of MnSOD prevents murine fibrosarcoma (FsaII) cells from apoptosis and promotes a differentiation program upon treatment with 5-azacytidine: Involvement of MARK and NF-kB pathways. *Antioxidants Redox Signal.* **3,** 375–385.
10. Zhao, Y., Xue, Y., Oberley, T. D., Kiningham, K. K., Lin, S.-M., Yen, H. C., et al. (2001) Overexpression of manganese superoxide dismutase suppresses tumor formation by modulation of activator protein-1 signaling in a multistage skin carcinogenesis model. *Cancer Res.* **61,** 6082–6088.
11. Fischer, S. M., Jasheway, D. W., Klann, R. C., Butler, A. P., Patrick, K. E., Baldwin, J. K., and Cameron, G. S. (1989) Correlation of phobol ester promotion in the resistant C57BL/6J mouse with sustained hyperplasia but not ornithine decarboxylase or protein kinase C. *Cancer Res.* **49,** 6693–6699.
12. Iversen, O. H. (1995) Of mice and men: a critical reappraisal of the two-stage theory of carcinogenesis. *Crit. Rev. Oncology* **6,** 357–405.
13. Troll, W. (1991) Prevention of cancer by agents that suppress oxygen radical formation. *Free Radic. Res. Commun.* **12–13,** 751–757.

14. Slaga, T. J. (1995) Inhibition of the induction of cancer by antioxidants. *Adv. Exp. Med. Biol.* **369,** 1671–1674.
15. Kouzarides, T. and Ziff, E. (1988) The role of the leucine zipper in the Fos-Jun interaction. *Nature* **336,** 646–651.
16. Gopalakrishna, R. and Anderson, W. B. (1989) Ca^{2+}- and phospholipid-independent activation of protein kinase C by selective oxidative modification of the regulatory domain. *Proc. Natl. Acad. Sci. USA* **86,** 6758–6762.
17. Nishizuka, Y. (1988) The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature* **334,** 661–665.
18. Palumbo, E. J., Sweatt, J. D., Chen, S.-J., and Klann, E. (1992) Oxidation-induced persistent activation of protein kinase C in hippocampal homogenates. *Biochem. Biophys. Res. Comm.* **187,** 1439–1445.
19. McSwine-Kennick, R. L., McKeegan, E. M., Johnson, M. D., and Morin, M. J. (1991) Phorbol diester-induced alterations in the expression of protein kinase C isozymes and their mRNAs. *J. Biol. Chem.* **266,** 15,135–15,143.
20. Verma, A. K., Pong, R. C., and Erickson, D. (1986) Involvement of protein kinase-C activation in ornitine decarboxylase gene expression in primary culture of newborn mouse epidermal cells and in skin tumor promotion by 12-*O*-tetradecanoylphor-bol-13 acetate. *Cancer Res.* **46,** 6149–6155.
21. Karin, M. (1995) The regulation of AP-1 activity by mitogen-activated protein kinases. *J. Biol. Chem.* **270,** 16,483–16,486.
22. Yen, H. C., Oberley, T. D., Vichitbandha, S., Ho, Y. S., and St. Clair, D. K. (1996) The protective role of manganese superoxide dismutase against adriamycin-induced acute cardiac toxicity in transgenic mice. *J. Clin. Invest.* **98,** 1253–1260.
23. Ibrahim, H., Lee, U.-S., Yen, H. C., St. Clair, D. K., and Chow, C. K. (2000) Antioxidant and oxidative status in tissues of managanese superoxide dismutase transgenic mice. *Free Radic. Biol. Med.* **28,** 379–402.

15

Fibroblastic, Hematopoietic, and Hormone Responsive Epithelial Cell Lines and Culture Conditions for Elucidation of Signal Transduction and Drug Resistance Pathways by Gene Transfer

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Abstract

Elucidation of signal transduction pathways involved in proliferation, cell cycle progression and the regulation of apoptosis has shown great promise in the treatment of various diseases including neoplastic, inflammatory, autoimmune, immunodeficiency, arthritic and neurodegenerative disorders. By understanding how these signal transduction pathways function, chemotherapeutic targets may be identified which will suppress or eliminate the disease. This information may eventually be translated into therapy, which would either eliminate or safely contain the patient's disease. This chapter will focus on basic tissue culture techniques which are used to elucidate signal transduction pathways. Furthermore, this chapter will provide a general background for understanding how gene transfer techniques can be used to elucidate signal transduction pathways as well as various pitfalls commonly encountered with their usage.

Key Words: Oncogenes; gene transfer; conditional constructs; Raf; MEK; PI3K; Akt; protein kinases; signal transduction inhibitors; hematopoietic cells; breast cells; fibroblast cells.

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1. Introduction

Much of our knowledge regarding signal transduction and apoptotic pathways has been derived from studies initiated to determine the function of virally encoded and cellular-derived oncogenes. Studies dating back to the 1970s and 1980s resulted in the determination that the *v-Src* oncogene encoded a protein tyrosine kinase (PTK) and *v-Ha-Ras* encoded a GTP/GDP exchange protein. The Rosetta Stone for understanding the nature of oncogenes encoded by RNA tumor viruses, excluding HTLV-I and II, was the discovery that the viral oncogenes contained in acute retroviruses had cellular homologs. This observation provided much of the physiological background and enthusiasm to determine the functions of other viral and cellular-encoded oncogenes. Propelled by these initial studies with virally encoded oncogenes, it was observed that many of the common chromosomal translocations identified in cancer patients often involved a cellular oncogene fused with another gene that would alter their biological activities. Chromosomal translocations were determined to occur between many different types of genes, including: 1) between genes shown to be homologous to viral oncogenes and known cellular genes [e.g., c-*myc* and the immunoglobulin (Ig) heavy and light chain genes in Burkitt's lymphoma in which the Ig gene provides a strong enhancer and the translocation may delete part of the c-*myc* promoter]; 2) between known oncogenes and unknown cellular genes [e.g., c-*abl* and *bcr* in the BCR-ABL translocation in chronic myelogenous leukemia in which the translocation disrupts both the c-*abl* and *bcr* genes]; and finally; 3) between genes whose roles in cancer were poorly known [e.g., TEL-JAK in which the TEL oncogene provides an oligomerization domain which results in constitutive JAK kinase activity in the absence of ligand]. These oncogenes have allowed investigations to determine the basis of how some cells become malignantly transformed. Functions of these oncogenes and important regulatory sequences have been determined by gene transfer experiments similar to those described in the next two chapters. It is through transformation with recombinant genes encoding various modifications that the complexity and interrelationships of various signal transduction pathways have been defined. Through the use of modified genes contained in readily transferable vectors, an investigator can begin to unravel a signaling pathway and potentially identify sites that may be sensitive to inhibition by chemotherapeutic drugs or other approaches to suppress or eliminate cell growth.

1.1. Basic Signal Transduction Pathways

There are multiple signal transduction pathways, which are involved in transmitting various types of signals including growth, differentiation, death or senscence without cell death (*see* **Fig. 1**). These signal transduction pathways can

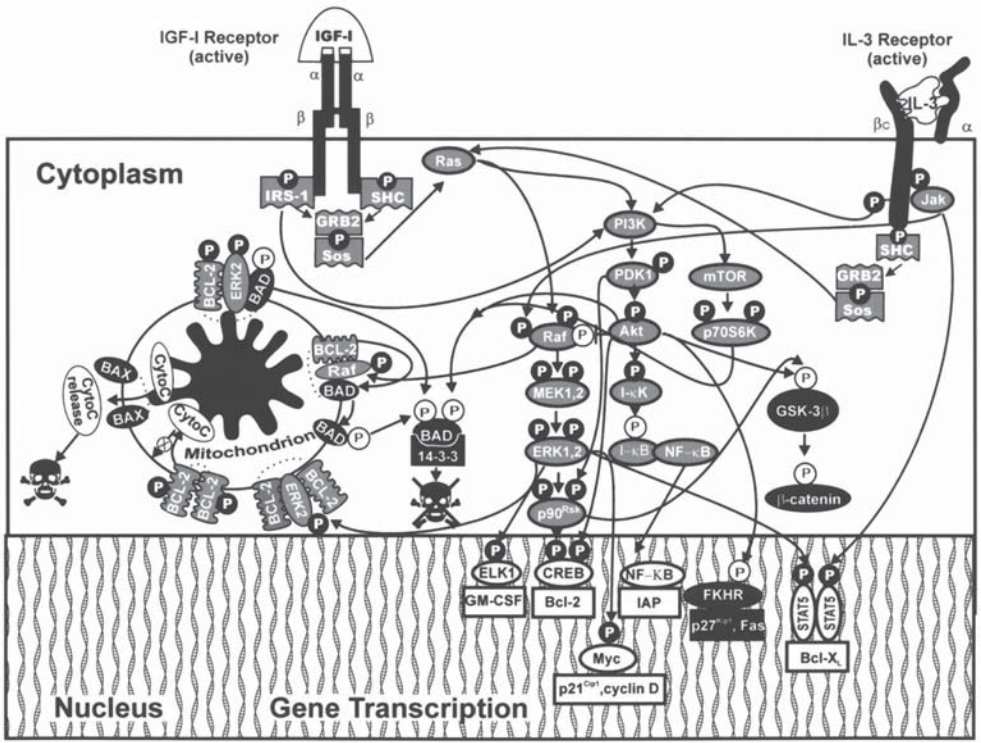

Fig. 1. Overview of cytokine-mediated signal transduction. Activation of kinase cascades by cytokine and growth factors. Cytokines and growth factors can activate Ras which subsequently activates Raf/MEK/ERK or PI3K/Akt cascades. Phosphate residues associate with activation and indicated with an black circle and a white P. Phosphate residues associated with inactivation are associated with a clear circle and a black P. Potential target genes modulated by signal transduction pathways activated by IL-3 and IGF-I such as GM-CSF, Bcl-2, Bcl-X_L, $p27^{Kip1}$, $p21^{Cip1}$, cyclin D, Fas, and IAP (inhibitor of apoptosis) are indicated.

become abnormally regulated in many disease states (e.g., neoplastic, autoimmune, and neurodegenerative diseases just to name a few). In this **Subheading**, we discuss three signal transduction pathways prominently involved in regulation of growth and apoptosis in hematopoietic and other types of cells, which are *Jak*/STAT, *Raf*/MEK/ERK, and PI3K/*Akt* cascades. We will provide an overview of these three pathways because they will be used as models to illustrate how signal transduction pathways can be characterized by wild type (WT), conditionally active (CA), or conditional (Cond) oncogenes.

1.2. Jak/STAT Pathway

The *Jak*/STAT pathway is a commonly used signal transduction pathway in hematopoietic and other types of cells ***(1–5)***. It is activated by many cytokines and growth factors. Jak proteins often serve to transmit signals from receptors that lack intrinsic kinase activity such as the IL-3R. The Jak proteins are tyrosine kinases, which become activated after interleukin-3 (IL-3) receptor dimerization. Activated Jak proteins phosphorylate STAT transcription factors, which control the expression of many genes including cytokine-inducible sequence (CIS), *Bcl-*X_L, $p21^{Cip1}$, cyclin D, oncostatin M, and leukemia inhibitory factor (LIF). Both Jak and STAT molecules are members of multigene families, which currently consist of at least four Jak and seven STAT family members. Moreover, CIS proteins are a multigene family that consists of at least eight family members. Different CIS proteins prevent activation of Jak kinases by binding the domain on the cytokine receptor normally occupied by the Jak molecule. CIS proteins can also bind to Jak proteins to inhibit their activity directly. Thus, in a simple sense, CIS family members provide negative feedback to limit effects of activated Jak kinases.

1.3. Ras/Raf/MEK/ERK Pathway

This is a central pathway activated by mitogens and growth factors. We will discuss activation of this pathway by cytokines and growth factors such as IL-3 and insulin growth factor (IGF-1). After IL-3 or IGF-1 receptor ligation, Shc, an SH2-domain containing protein, associates with the *c*-terminus of the IL-3R β_c-chains or IGF-1R ***(1–5)*** (*see* **Fig. 1**). Shc recruits the GTP-exchange complex GRB2/SOS resulting in the loading of membrane bound Ras with GTP ***(1–5)***. Ras:GTP then recruits Raf to the membrane where it becomes activated by tyrosine (Y) and serine (S)/threonine (T) kinases. Moreover, certain phosphates on *Raf* are removed by phosphatases such as protein phosphatase 2A (PP2A), which can contribute to *Raf* activation. There are at least six residues on Raf, which can be phosphorylated or dephosphorylated, and these phosphorylation events have either positive or negative effects on Raf activity depending upon which sites are phosphorylated ***(1–4)***. Raf is responsible for phosphorylation and activation of

the mitogen associated/extracellular regulated kinase-1 (MEK1) on S218 and S222 ***(1–5)***. MEK1 phosphorylates and activates extracellular regulated kinases 1 and 2 (ERKs 1 and 2) on specific T and Y residues (T202 and Y204 on ERK1, T185 and Y187 on ERK2) ***(1–5)***. Activated ERK1 and ERK2 S/T kinases phosphorylate and activate a variety of substrates including $p90^{Rsk1}$ ***(1–5)***. $p90^{Rsk1}$ can phosphorylate and activate cyclic-AMP response element binding protein (CREB) ***(5)***. Moreover, ERK can translocate into the nucleus and phosphorylate additional transcription factors such as Elk1, which binds promoters of many genes including IL-3 and GM-CSF ***(1)***.

1.4. PI3K/Akt Pathway

The phosphatidyl-inositol 3 kinase (PI3K)/Akt pathway is a complex pathway that controls apoptosis and cell cycle progression ***(1–5)***. PI3K can become activated by binding of the regulatory p85 PI3K subunit to a specific residue on the IL-3 receptor ***(1–5)***. This binding results in the activation of the p110 PI3K catalytic subunit. IGF-1 also activates the PI3K pathway either via GRB2/SOS or through the insulin receptor substrate-1 (IRS-1). Activated PI3K phosphorylates the membrane lipid phosphatidyl-inositol (4,5)-bisphosphate [PtdIns(4,5)P_2] to form phosphatidyl-inositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P_3], which activates PI3K dependent kinase (PDK1). PDK1 then phosphorylates the S/T kinase Akt at T308.

Akt can transduce an anti-apoptotic signal by phosphorylating downstream target proteins involved in the regulation of cell growth [e.g., glycogen synthetase kinase-3β (GSK-3β), pro-apoptotic Bad, and the forkhead family transcription factor FKHR-L1] ***(6–9)***. Phosphorylated FKHR-L1 loses its ability to induce expression of *Fas* and $p27^{Kip1}$ genes, which are associated with cell death (necrosis) and inhibition of cell cycle progression ***(4–5)***. Akt also phosphorylates I-κK, which subsequently phosphorylates I-κB, an inhibitor of the transcription factor NF-κB resulting in its ubiquitination and subsequent degradation in proteosomes ***(4–5)***. Disassociation of I-κB from NF-κB enables NF-κB to translocate into the nucleus to promote gene expression that, under certain circumstances, stimulates growth and prevents apoptosis ***(4–5)***. The PI3K/Akt pathway can also phosphorylate and activate CREB, which regulates transcription of antiapoptotic genes such as Bcl-2 and GM-CSF ***(1–5)***.

GSK-3β phosphorylates cyclin D (T286), c-*myc* (T58) and β-catenin and targets these proteins for degradation ***(6)***. Stabilization or overexpression of β-catenin is associated with cell transformation ***(4–7)***. Abrogation of GSK-3β kinase activity resulting from Akt phosphorylation would be expected to have prosurvival effects because β-catenin would not be targeted for degradation. Accumulation of cyclin D would be expected to lead to the phosphorylation of the retinoblastoma protein (pRB) and cell-cycle progression. Thus, phosphorylation and

inactivation of GSK-3β can have growth-promoting effects. Interestingly, phosphorylation of GSK-3β can be mediated via other pathways including the Raf/MEK/ERK pathway that activates $p90^{Rsk1}$, which is another GSK-3β kinase ***(4–7)***.

The PI3K pathway also activates ribosomal protein kinases such as $p70^{S6K}$ (an S6 ribosomal protein kinase). $p70^{S6K}$ enhances translation of certain mRNAs, is needed for the early events of cell cycle progression and suppresses apoptosis by phosphorylating Bad ***(6)***.

The PI3K pathway is negatively regulated by phosphatases. PTEN (phosphatase and tensin homolog deleted on chromosome 10, a.k.a., MMAC1 [mutated in multiple advanced cancers]) is considered a tumor suppressor gene ***(3–5)***. PTEN is a dual specificity lipid and protein phosphatase that removes the 3-phosphate from the PI3K lipid product $PtdIns(3,4,5)P_3$ to produce $PtdIns(4,5)P_2$, which prevents Akt activation.

1.5. Interactions Between the Jak/STAT, Ras/Raf/MEK/ERK and Ras/PI3K/Akt Pathways

Interactions between Jak/STAT and Raf/MEK/ERK pathways occur, including enhanced Jak activity by ERK phosphorylation and Jak stimulation of the Raf cascade ***(1)***. Signaling pathways such as Jak/STAT, *Raf*/MEK/ERK, and PI3K/*Akt* cascades modulate expression of cyclins, cyclin-dependent kinases (Cdks), and Cdk inhibitors through downstream transcription factors. For instance, activation of *Raf*/MEK/ERK or PI3K/*Akt* pathways each induces cyclin D expression ***(1)***. Moreover, activation of these cascades together leads to synergistic activation of cyclin D expression ***(1)***.

Recently, it was shown that Akt could phosphorylate *Raf* on S259 and lead to inactivation of *Raf* in the MCF-7 breast cancer line and differentiated myotubules, but not in their myoblast precursor cells ***(5)***. Akt has also been shown to phosphorylate B-*Raf*, which results in its inactivation ***(5)***. The Akt-related kinase serum/glucocorticoid regulated kinase (SGR) can phosphorylate B-Raf to inhibit its cell cycle inhibitory and proapoptotic activities ***(5)***. However, the fact that Raf has at least six phosphorylation sites should be kept in mind and these results may not be valid in all cell types. Moreover, these kinases may not be the physiologically relevant kinases that phosphorylate *Raf-1* and B-*Raf*. Studies in 32D hematopoietic cells have shown that *Akt* can activate *Raf-1* on both the plasma and mitochondrial membranes through a Ras-independent but PKC dependent mechanism. This latter mechanism is associated with cell survival ***(5)***. Thus, Akt and related proteins phosphorylate Raf family members and either inhibit or enhance their activity. In addition, suppression of apoptosis in some cells by *Raf* and MEK requires PI3K activity ***(5)***. Thus, these three signal transduction pathways can interact and cross regulate each other. However, the above experiments illustrate the caution that must be applied to gene-transfer

experiments. Just because a kinase can phosphorylate a tempting substrate in cells that overexpress the kinase does not mean this event is necessarily relevant in vivo.

2. Materials

2.1. Common Cell Lines Used for Elucidating Functions of Genes Involved in Signal Transduction, Apoptosis, and Drug Resistance

The cell line one uses to study effects of a gene is often dictated by the particular cell system the investigator is interested in. Additionally, many investigators are more interested in the functions of particular oncoproteins than investigating them in the physiologically relevant context of the type of cell transformed by the oncogene. This often creates problems as certain cell lineage-specific oncogenes will have different effects in different cell types.

2.2. NIH-3T3 Cells

The NIH-3T3 cell line is one of the most common cell lines used for functional analysis of proteins, including oncoproteins. This is a relatively standard fibroblastic and adherent cell line that does not readily induce tumors upon injection into immunocompromised mice. This murine fibroblastic cell line readily grows in tissue culture with medium supplemented with 5–10% fetal bovine serum (FBS) or iron-supplemented defined bovine calf serum (BCS). BCS from Hyclone (Logan, UT) is less expensive than FBS. NIH-3T3 cells will remain nontransformed as long as the cell density and passage number are closely monitored. As their name implies, NIH-3T3 cells should be transferred after trypsin treatment 1 to 3 every 3 d (*see* **Notes 1–2**). Media that can be used to culture NIH-3T3 cells includes minimal essential Eagle's medium (MEM), Dulbecco's modified Eagle's medium (DMEM), and Iscove's modified Dulbecco's medium (IMDM) containing 5–10% heat inactivated FBS or BCS. Most media can be purchased as powder mixtures from Invitrogen (San Diego, CA) and the investigator can make 1X medium using purified water. The investigator will also have to add L-glutamine (100X stock is 29.2 g/L = 200 m*M*), sodium pyruvate (a 100X stock is 22 g/L = 200 m*M*), sodium penicillin-G (200X stock is 6 g/L = 17 m*M*), streptomycin sulfate (200X stock is 10 g/L = 7 m*M*), and 500-mL heat-inactivated FBS or BCS to the medium (10 L total). Some media may require 50 µ*M* 2-mercaptoethanol, which can be made as a 1000X stock (1.75 mL of 14.3 *M* 2-mercaptoethanol diluted to 500 mL with water) that should be filter sterilized, wrapped in foil because it is light sensitive, and stored at 4ºC. Media should be filter sterilized, then stored in a refrigerator or a cold room. Some investigators will wrap media with foil because some components are light sensitive. Moreover, if one

is not going to rapidly use the medium, one may want to add antibiotics and L-glutamine from a 100X stock solution, which can be purchased from Invitrogen (*see* **Note 3**).

2.3. Rat-1 Cells

Even though NIH-3T3 cells are by definition not transformed because they do not form tumors upon injection into nude mice or form foci in soft agar, they possess mutated growth regulatory genes that make them more susceptible to transformation by activated oncogenes than many other fibroblast cell lines. Transformation of Rat-1 cells requires gain of at least two oncogenes that collaborate with one other. This line, as well as primary rat fibroblasts, revealed that malignant transformation often involves mutations at multiple oncogenes. This cell line grows in standard tissue culture medium (MEM, DMEM, or IMEM) containing 10% FBS.

2.4. PA317, ψ2, ψAM, gp+E86, BOSC23, Phoenix, and 293T Retroviral Packaging Cell Lines

These are seven commonly used retroviral packaging cell lines. Some contain the necessary Moloney Murine Leukemia Virus (Mo-MuLV) genes for packaging the recombinant retrovirus, but lack *cis* acting retroviral packaging (ψ) sequences necessary for retroviral genome encapsulation (PA317, φ2, φAM, and gp+E86). Thus, the preexisting Mo-MuLV genome cannot be packaged into a virion. In contrast, the genomic RNA encoded by the recombinant retrovirus containing ψ sequences can be packaged into a retroviral virion. The recombinant retrovirus will proceed through one and only one round of infection, providing that recipient cells do not contain any endogenous replication competent type C retroviruses. Certain retroviral packaging cell lines such as gp+E86 contain the retroviral glycoprotein and envelope sequences on different pieces of DNA (different chromosomes) to reduce the possibility of recombination of the recombinant retroviral genome with *gag* and *env* sequences present in the integrated helper genome. The human 293T is an embryonic kidney cell line, containing simian virus T antigen, which requires cotransfection with the retroviral sequences present on two separate plasmids for expression of the introduced recombinant retrovirus. These cell lines grow in standard tissue culture media (MEM, DMEM, or IMDM) with 5–10% FBS or BCS.

2.5. WI-38 Human Fibroblastic Cell Line

WI-38 is a model of non-transformed human cells. These cells can be difficult to grow in culture and often have a long doubling time. This cell line grows in standard tissue culture medium (MEM, DMEM, IMDM) with 10–20% FBS and usually high glucose.

2.6. FDC-P1, FL5.12, Ba/F3, 32Dc13, and NFS-70 Murine Hematopoietic Cell Lines

These cytokine-dependent murine cell lines require IL-3 or granulocyte/macrophage colony stimulating factor (GM-CSF) in addition to the growth factors present in FBS or BCS for proliferation *(1,8–22)*. These cell lines are not normally tumorigenic upon injection into immunocompromised mice and serve as good models to investigate the function of various genes involved in signal transduction and apoptosis in hematopoietic cells (*see* **Note 4**).

2.7. TF-1 Human Hematopoietic Cell Line

The TF-1 hematopoietic cell line was derived from a patient with an erythroleukemia and requires either GM-CSF or IL-3 to proliferate *(1)*. This cell line is responsive to other cytokines including erythropoietin (epo), IL-5, IL-11, Kit-L, and thrombopoietin *(19–21)* (*see* **Note 5**).

2.8. MO7-E, OCI-AML5, and AML193 Human Hematopoietic Cell Lines

These human cell lines are either growth factor dependent (MO7-E) or growth factor responsive (OCI-AML5 and AML193) and were derived from human leukemia biopsies *(1,25)*. The MO7-E cell line will proliferate in response to either IL-3 or GM-CSF and will also respond to IL-9 and other cytokines (*see* **Note 6**).

2.9. Jurkat, HL-60, K562, and U937 Human Hematopoietic Cell Lines

These transformed human hematopoietic cell lines grow in media (Roswell Park Memorial Instiutute [RPMI]-1640, IMDM, or DMEM) supplemented with 5–10% FBS or BCS in the absence of additional growth factors (*see* **Note 7**).

2.10. EL4, BW5147, and WEHI-3B Murine Cell Lines

These transformed murine hematopoietic cell lines are often used as models or controls to investigate gene expression in murine hematopoietic cell lines *(1)*. All three cell lines grow in media (RPMI-1640, IMDM, or DMEM) supplemented with 5–10% FBS or BCS and do not require additional growth factors for their proliferation *(28–30)* (*see* **Note 8**).

2.11. MCF-7 Human Breast Cancer Cell Line

The MCF-7 (Michigan Cancer Foundation) cell line is an estrogen receptor positive breast cancer cell line, which represents a good model to investigate the effects of various oncogenes on signal transduction pathways functioning in breast cancer cells *(31–36)*. This cell line grows in RPMI-1640 with 5–10% FBS. Adherent MCF-7 cells should be passed after trypsin treatment (*see* **Note 9**).

2.12. LNCaP Human Prostate Cancer Cell Line

The LNCaP cell line is an androgen receptor positive prostate cancer cell line that grows in RPMI-1640 with 10–20% FBS (*see* **Notes 10–11**). Adherent LNCaP cells should be passed after trypsin treatment.

2.13. Common Solutions and Equipment Used to Measure Cell Growth

1. Trypan blue dye solution: 1 mL of 10X trypan blue stock (0.4% trypan blue in 0.81% sodium chloride and 0.06% dibasic potassium phosphate, Sigma) is diluted with 9 mL of PBS, then filter sterilized.
2. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide] (Sigma).
3. [^{3}H]-Thymidine (New England Nuclear, Boston, MA).
4. Hemacytometer and cover slips.
5. Coulter counter to count cells.

3. Methods

3.1. Culture of NIH-3T3 Fibroblast Cells

This murine fibroblastic cell line readily grows in tissue culture with medium supplemented with 5–10% FBS or BCS (Hyclone, Logan, UT). NIH-3T3 cells will remain nontransformed as long as the cell density and passage number are closely monitored. Cells can be grown either in Petri dishes (60, 100, or 150 mm, Corning, Corning, NY) for tissue culture or tissue culture flasks (T25, T75, or T150 cm) depending upon the number of cells desired. When cells reach approx 75–100%, medium should be removed by aspiration with a Pasteur pipet attached to a vacuum source and the cell monolayer should be washed once with PBS followed by 1X trypsin treatment for approx 5 min at 37ºC in a 5% CO_2 incubator. The solution containing trypsinized cells should be centrifuged at 1000*g* for 5–10 min. Trypsin solution can be removed from the centrifuge tube by aspiration with a Pasteur pipet (be sure not to aspirate the cell pellet). The investigator should make sure that the cell pellet is at the bottom of the centrifuge tube as sometimes after trypsin treatment the cells form aggregates and "float" as a cloudy suspension that may be lost if the trypsin solution is decanted or aspirated too quickly from the centrifuge tube. As their name implies, NIH-3T3 cells should be transferred after trypsin treatment 1–3 every 3 d.

3.2. Heat Inactivation of Serum

FBS and BCS should be heat-inactivated before use, to kill certain viruses in the serum and to inactivate complement. This is done by placing a bottle containing the serum (100 or 500 mL) in a 56ºC water bath for 30 min. The bottle should be swirled gently to resuspend the serum components before placing it in

the water bath. Care should be taken when heat-inactivating serum as one should make sure that the temperature does not exceed 56ºC and time of inactivation does not exceed 30 min. A thermometer should be kept in the water bath, as temperature control in some water baths is not reliable. Excessive heat inactivation destroys various critical growth factors in the serum necessary for cell growth.

3.3. Culture of Hematopoietic Cytokine Dependent Cells

These cell lines grow in tissue culture medium (IMDM, DMEM, or RPMI-1640) with 5–10% FBS or BCS and 5–20% conditioned WEHI-3B supernatant (economical source of murine IL-3) or other cytokines.

The WEHI-3B cell line has an intracisternal type A particle transposed in the IL-3 gene, which results in constitutive synthesis of IL-3. WEHI-3B supernatant is prepared by growing WEHI-3B cells in DMEM with 5% BCS in large tissue culture flasks (T150 cm) until the cells become dense (2×10^6/mL, or many cells loosely adhere to the bottom of the flask and form a "loose" monolayer). Medium containing cells is poured into large Corning centrifuge tubes (250 mL conical tubes) and centrifuged at 4000*g* for 10 min. The supernatant is decanted and either filter sterilized (0.2 µm bottle top filters, Corning, NY) or stored at –20ºC to be processed later. Fresh medium is fed back to flasks containing WEHI-3B cells and approximately every 3 d a new preparation of WEHI-3B supernatant can be prepared. Murine IL-3 in the WEHI-3B supernatant is stable at –20ºC for years. The amount of IL-3 in the WEHI-3B supernatant can be determined by titering the supernatant by adding serial dilutions of supernatant to sensitive IL-3 dependent cells such as FDC-P1, FL5.12, or 32Dcl3 cells, then performing [^{3}H]-thymidine incorporation or MTT proliferation assays.

3.4. Assays of Growth by [^{3}H]-Thymidine Incorporation

Supernatant containing growth factor is plated in triplicate and a series of threefold dilutions is made in a 96-well flat bottom plate (Corning). Threefold dilution is performed by transferring 50 µL into 100 µL of medium lacking cytokines but containing either FBS or BCS and subsequently removing 50 µL and transferring to the next row in the plate with a multichannel pippetor. This is performed down the remaining seven rows of the plate. Indicator cells are washed twice with PBS to remove any residual growth factors, then plated at 1×10^4 cells/well in 100 µL of medium (200 µL total). Murine hematopoietic cells (FDC-P1, FL5.12, or 32Dc13) are cultured for 1 d in the absence or presence of different dilutions of the supernatant or recombinant cytokine. In contrast, human hematopoietic cells (TF-1 and MO7E) are cultured for 3 days in the same conditions. Proliferation is estimated by assaying incorporation of [^{3}H]-thymidine (6.7 Ci/mmol, NEN, Boston, MA), which is added during the last 4–6 h of culture as described ***(8–25)***. Recombinant cytokines are used as controls to determine the levels of

cytokines present in the supernatants. Supernatant cytokines are identified by the addition of neutralizing antibodies to the cytokines. Antibodies are mixed with supernatants or recombinant cytokines and incubated 30 min before addition of indicator cell lines. Different dilutions of the antibodies should be used to titrate the antibody.

3.5. MTT Assay

MTT is a water soluble reagent useful for rapid quantitation of viable adherent or nonadherent cells in 96 well plates. The tetrazolium ring of MTT is cleaved by mitochondrial dehydrogenases in viable cells to yield a water insoluble purple formazan product. MTT based viability assays may be used to measure either growth or cytotoxicity resulting from either drug treatment or ectopic gene expression after transfection or retroviral infection. Cells should be seeded at a density of 1000 to 10,000 cells/well in 100 to 200 µL of media prior to any experimental treatments, if any. For quantitation of viable cells, one tenth volume of 5 mg/mL MTT stock dissolved in an appropriate tissue culture medium is added to each well to produce a final MTT concentration of 500 µg/mL. Cells are incubated for 2 to 4 h at 37ºC to allow formation of purple formazan crystals from MTT cleavage. Nonadherent cells are centrifuged at 500*g* for 5 min at 4ºC to pellet formazan crystals at the bottom of the wells. Centrifugation is not necessary for adherent cells. Media is removed by aspiration, then formazan crystals are dissolved in 100 to 200 µL of dimethyl sulfoxide (DMSO) for 5 min at 37ºC. Absorbance is measured with a plate reader at 540 nm. MTT treatment of wells containing media alone should be used for reference absorbance to account for any absorbance in the absence of cells. Determination of a standard curve will indicate the range that absorbance at 540 nm is proportional to the amount of viable cells. The volume of DMSO added should be adjusted to ensure absorbance values fall within this range.

3.6. Culture of Hematopoietic Cells with Recombinant Growth Factors

Recombinant murine IL-3 (1 ng/mL) or GM-CSF (1 ng/mL) can be used to culture murine IL-3/GM-CSF dependent cells. Human TF-1 and MO7E cell lines grow in tissue culture media (IMDM or RPMI-1640) with 5 to 10% FBS and 1 ng/mL recombinant human IL-3 or GM-CSF. Recombinant cytokines can be purchased from many sources including R&D Systems (Minneapolis, MN). Cytokines can be diluted with tissue culture medium or PBS and should be aliquoted and kept at −20ºC or −80ºC. For prolonged storage, it is better if cytokines are dissolved in medium with bovine serum albumin to improve cytokine stability.

4. Notes

1. NIH-3T3 cells should not be allowed to remain at 100% confluence for more than 1–3 d (some references even say 1 d), unless one is trying to isolate transformed cells. Cells should be frozen down after the investigator has received them and should not be passed for greater than ten passages (1–2 mo).
2. Although the NIH-3T3 fibroblastic cell line has been pivotal to the elucidation of the functions of many genes, it may not be appropriate for the understanding of genes that encode more tissue specific functions (e.g., hematopoietic specific oncogenes).
3. If one is not going to use a lot of a particular medium, one may want to purchase that medium already made. Moreover, one has to be careful that the H_2O used does not contain endotoxins or other substances, which may inhibit the growth of the cell line. Finally, one has to be careful that the glassware one uses is truly clean and does not contain residual detergents, which will inhibit cell growth. Significant problems can arise from each of these different issues. Thus in some cases it may be more effective to use plastic disposable pipettes, tissue culture flasks, bottles and Erlenmeyer flasks, tissue culture medium, and PBS from a reputable source.
4. The FDC-P1 cell line is more susceptible to transformation by activated oncogenes than other murine hematopoietic cell lines, which may result in part from mutation of certain tumor suppressor genes ***(1,11–14,19)***. The FL5.12 cell line is less susceptible to transformation by activated oncogenes such as *Src*, *Raf*, and others ***(1,8,9,15–18)***. FL5.12 cells may require activation of more than one signal transduction pathway to result in abrogation of cytokine dependence ***(8)***. The identification of the anti-apoptotic function of the Bcl-2 oncogene was first identified in this cell line ***(1)***. The Ba/F3 cell line is similar to the FL5.12 cell line and both were derived at the Basel Institute for Immunology at approximately the same time period (1984–1985). The BaF3 cell line has been used to determine the functions of many cytokine receptor domains (e.g., IL-3Rβ and IL-2Rβ chains) ***(1)***. 32Dc13 cells can be induced to differentiate into more granulocytic cells when cultured in the presence of G-CSF instead of IL-3. 32Dc13 cells are used as a model to investigate effects of oncogenes on differentiation of hematopoietic cells because certain activated oncogenes such as v-Abl block differentiation of these cells. Cytokine withdrawl causes apoptosis by these cells within 24 h. Certain oncogenes will abrogate cytokine dependence and, hence, prevent apoptosis in these cells ***(9–15)***. These hematopoietic cells are not readily transfected with commercial liposomes but can be transfected by electroporation or retroviral infection ***(16–18)***.
5. The TF-1 cell line can be induced to differentiate into more erythroid like cells by epo (1 ng/mL), hemin (40 μ*M*, Sigma), and delta aminolevulinic acid (0.5 m*M*, δ-ALA, Sigma) or into more macrophage like cells by 10 to 50 n*M* phorbol myristic acid (PMA) ***(1)***. This cell line represents a good model to examine effects of activated oncogenes in human hematopoietic cells, which often respond to multiple hormones ***(6,20–24)***. Furthermore, these cells represent a good source to examine the effects of various chemotherapeutic agents (e.g., doxorubicin, taxol, and geldanamycin) on cell death and differentiation. Moreover, effects of various novel compounds such as diptheria toxin conjugated IL-3 on apoptosis in cytokine dependent and oncogene transformed cells has been determined in these cells ***(23)***.

6. The cytokine dependent MO7-E cell line has been used to investigate effects of the *BCR-ABL* oncogene on the induction of malignant transformation and autocrine growth factor synthesis ***(1)***. The OCI-AML5 is a relatively unique cell line in that it is responds to multiple cytokines including IL-3, G-CSF, GM-CSF, the ligand for the c-Kit receptor (Kit-L), and importantly the Flt-3 receptor ligand (FL). Most other hematopoietic cell lines are not responsive to this early acting hematopoietic growth factor. Proliferation of the OCI-AML5 cell line is enhanced upon addition of IL-3, GM-CSF, Kit-L, and G-CSF. The OCI-AML5 cell line should not be confused with the OCI-AML3 cell line, which readily grows in the absence of exogenously supplemented hematopoietic growth factors. The AML193 cell line is also responsive to IL-3 and GM-CSF. Because the OCI-AML5 and AML193 cell lines are not 100% factor dependent, they do serve as sources to understand effects of activated oncogenes on proliferation of hematopoietic cells.
7. The Jurkat T-cell line contains rearranged T-cell receptor genes and represents a model to investigate signal transduction pathways in T cells. This cell line has recently been shown to lack the important PTEN tumor suppressor protein and thus may have a hyperactive PI3K/Akt pathway ***(26)***. Although this cell line is widely used by many investigators, caution must be used in determining which subline the investigator is working with as not all sublines behave in identical fashions. HL-60 is a granulocytic cell line, which can be induced to differentiate into more mature granulocytes after phorbol ester treatment or more mature erythroid cells after DMSO treatment ***(27)***. The K562 erythroleukemia cell line has the BCR-ABL chromosomal translocation and is often used as a tissue culture model to investigate some of the events involved in chronic myelogenous leukemia (CML). U937 is a monocytic cell line and can be induced with PMA to differentiate into more mature adherent monocytic cells. These cell lines can be readily transfected by retroviral vectors. However, they are more difficult to electroporate or transfect with various commercially available liposomes.
8. EL4 and BW5147 are both murine T-cell lines and can be induced to express multiple hematopoietic cytokines, (e.g., IL-2, IL-3, and GM-CSF). They can be used as controls for the expression of many hematopoietic specific genes for northern, western, and RT-PCR assays. The BW5174 cell line has been used as a fusion partner with primary T cells to produce T-cell hybridomas ***(28–30)***. The WEHI-3B cell line constitutively produces IL-3 and is an economical source for this cytokine, which can be used to grow large volumes of IL-3 dependent hematopoietic cells. These cell lines are not readily transfected by commercial liposomes but can be transfected by electroporation or retroviral infection ***(28–30)***.
9. The MCF-7 line should not be confused with the MCF-10A and MCF-12A cell lines, which represent "less transformed" breast cancer (cyst) cell lines as they were not derived from the same patient ***(31–34)***. Furthermore, it has recently been shown that the drug resistant MCF/Adr cell line was not derived from the MCF-7 cell line; the MCF/Adr cell line is now referred to as NCI-Adr-Res ***(34)***. mRNA transcripts for the multidrug resistance gene (*mdr*-1, a.k.a., Pgp) are readily detected by RT-PCR in NCI-Adr-Res, but not in MCF-7 cells. These cell lines can be infected with retroviral vectors and transfected by commercial liposomes ***(34–36)***.

10. LNCaP cells represent a good model to investigate the effects of various oncogenes on the induction of hormone independence and drug resistance in prostate cancer cells. They can be transfected by retroviral infection and liposomes. The cells tend to grow slow after being thawed out. They should be thawed out in one well of a 6- or 24-well plate in 1 to 3 mL of medium. They should be monitored for retention of androgen-responsiveness.
11. Hematopoietic, breast, prostate, and other cell types tend to grow in a density-dependent fashion. The degree of density dependence can vary. When thawing and plating out cells, it is better to put them in a small volume (1–2 mL for hematopoietic cells and 2–5 mL for fibroblast or epithelial cells) for at least the first 24 h of culture. Once the cells start producing autocrine growth factors necessary for proliferation, growth will increase significantly.

References

1. Blalock, W. L., Weinstein-Oppenheimer, C., Chang, F., Hoyle, P. E., Wang, X. Y., Algate, P. A., et al. (1999) Signal transduction, cell cycle regulatory, and anti-apoptotic pathways regulated by IL-3 in hematopoietic cells: possible sites for intervention with anti-neoplastic drugs. *Leukemia* **13,** 1109–1166.
2. McCubrey, J. A., May, W. S., Duronio, V., and Mufson, A. (2000) Serine/threonine phosphorylation in cytokine signal transduction. *Leukemia* **14,** 9–21.
3. Wang, X. Y., Steelman, L. S., and McCubrey, J. A. (1997) Abnormal activation of cytokine gene expression by intracisternal type A particle transposition: effects of mutations which result in autocrine growth factor stimulation and malignant transformation. *Cytokines Cell Mol. Ther.* **3,** 3–19.
4. White, M. K. and McCubrey, J. A. (2001) Suppression of apoptosis: role in cell growth and neoplasia. *Leukemia* **15,** 1011–1121.
5. Lee, J. T. Jr. and McCubrey, J. A. (2002) The Raf/MEK/ERK signal transduction cascade as a target for chemotherapeutic intervention in leukemia. *Leukemia* **16,** (in press).
6. Dufner, A., Anjelkovic, M., Burgering, B. M. T., Hemmings, B., and Thomas, G. (1999) Protein kinase B localization and activation and eukaryotic translational initiation factor 4E-binding protein phosphorylation. *Mol. Cell. Biol.* **19,** 4525–4534.
7. Evan, G. I., Wyllie, A. H., Gilbert, C. S., Littlewood, T. D., Land, H., Brooks, M., et al. (1992) Induction of apoptosis in fibroblasts by c-myc protein. *Cell* **69,** 119–128.
8. McCubrey, J. A., Lee, J. T., Steelman, L. S., Blalock, W. L., Moye, P. W., Chang, F., et al. (2001) Interactions between the PI3K and Raf signaling pathways can result in the transformation of hematopoietic cells. *Cancer Detect. Prev.* **25,** 375–393.
9. McCubrey, J. A., Smith, S. R., Algate, P. A., DeVente, J. E., White, M. K., and Steelman, L. S. (1993) Retroviral infection can abrogate the factor-dependency of hematopoietic cells by autocrine and non-autocrine mechanisms depending on the presence of a functional viral oncogene. *Oncogene* **8,** 2905–2915.
10. McCubrey, J., Steelman, L. S., Wang, X.-Y., Davidian, E. W., Hoyle, P. E., White, C., et al. (1995) Autocrine growth factor secretion after transformation of human cytokine-dependent cells by viral and cellular oncogenes. *Int. J. Oncol.* **7,** 573–586.

11. Hoyle, P. E., Moye, P. W., Steelman, L. S., Blalock, W. L., Franklin, R. A., Pearce, M., et al. (2000) Differential abilities of the Raf family of protein kinases to abrogate cytokine-dependency and prevent apoptosis in murine hematopoietic cells by a MEK1-dependent mechanism. *Leukemia* **14,** 642–656.
12. Moye, P. W., Blalock, W. L., Hoyle, P. E., Chang, F., Franklin, R. A., Weinstein-Oppenheimer, C., et al. (2000) Synergy between Raf and BCL2 in abrogating the cytokine dependency of hematopoietic cells. *Leukemia* **14,** 1060–1079.
13. Blalock, W. L., Moye, P. W., Chang, F., Pearce, M., Steelman, L. S., McMahon, M., and McCubrey, J. A. (2000) Combined effects of aberrant MEK1 activity and BCL2 overexpression on relieving the cytokine-dependency of human and murine hematopoietic cells. *Leukemia* **14,** 1080–1096.
14. Blalock, W. L., Moye, P. M., Steelman, L. S., Franklin, R. A., McCarthy, S. A., Cherwinski, H., et al. (2000) A conditionally-active form of MEK1 results in autocrine transformation of human and mouse hematopoietic cells. *Oncogene* **19,** 526–536.
15. Blalock, W. L., Moye, P. M., Chang, F., Lee, J. T., Pohnert, S. C., Burrows, C., et al. (2001) Effects of inducible MEK1 activation on the cytokine dependency of lymphoid cells. *Leukemia* **15,** 794–807.
16. Mayo, M., Wang, X.-Y., Algate, P. A., Arana, G. F., Hoyle, P. E., Steelman, L. S., and McCubrey, J. A. (1995) Synergy between AUUUA motif disruption and enhancer insertion result in autocrine transformation of interleukin-3 dependent hematopoietic cells. *Blood* **86,** 3139–3150.
17. Wang, X. Y. and McCubrey, J. A. (1996) Malignant transformation induced by cytokine genes: a comparison of the abilities of germline and mutated interleukin 3 genes to transform hematopoietic cells by transcriptional and posttranscriptional mechanisms. *Cell Growth Differ.* **7,** 487–500.
18. Wang, X. Y. and McCubrey, J. A. (1997) Differential effects of retroviral long terminal repeats on interleukin-3 gene expression and autocrine transformation. *Leukemia* **11,** 1711–1725.
19. McCubrey, J. A., Steelman, L. S., Wang, X.-Y., Algate, P. A., Hoyle, P. E., White, C., et al. (1995) Differential effects of viral and cellular oncogenes on the growth factor-dependency of hematopoietic cells. *Int. J. Oncol.* **7,** 295–310.
20. McCubrey, J. A., Steelman, L. S., Hoyle, P. E., Blalock, W. L., Weinstein-Oppenheimer, C., Franklin R. A., et al. (1998) Differential abilities of activated Raf oncoproteins to abrogate cytokine-dependency, prevent apoptosis and induce autocrine growth factor synthesis in human hematopoietic cells. *Leukemia* **12,** 1903–1929.
21. Weinstein-Oppenheimer, C. R., Blalock, W. L., Steelman, L. S., Chang, F., and McCubrey, J. A. (2000) Raf signal transduction cascade as a target for chemotherapeutic intervention in growth factor-dependent tumors. *Pharmacol. Ther.* **88,** 229–279.
22. Weinstein-Oppenheimer, C., Steelman, L. S., Algate, P. A., Blalock, W. L., Burrows, C., et al. (2000) Effects of deregulated Raf activation on integrin, cytokine-receptor expression and the induction of apoptosis in hematopoietic cells. *Leukemia* **14,** 1921–1938.
23. Frankel, A. E., McCubrey, J. A., Miller, M. S., Delatte, S., Ramage, J., Kiser, M., et al. (2000) Diphtheria toxin fused to human interleukin-3 is toxic to blasts from patients with myeloid leukemias. *Leukemia* **14,** 576–585.

24. Hoyle, P. E., Steelman, L. S., and McCubrey, J. A. (1997) Autocrine transformation of human hematopoietic cells after transfection with an activated granulocyte/macrophage colony stimulating factor gene. *Cytokines Cell Mol. Ther.* **3,** 159–168.
25. McCubrey, J. A., Blalock, W. L., Saleh, O., Pearce, M., Burrows, C., Steelman, L. S., et al. (2001) Enhanced ability of daniplestim and myelopoietin-1 to suppress apoptosis in human hematopoietic cells. *Leukemia* **15,** 1203–1216.
26. Shan, X., Czar, M. J., Bunnell, S. C., Liu, P., Liu, Y., Schwartzberg, P. L., and Wange, R. L. (2000) Deficiency of PTEN in Jurkat T cells causes constitutive localization of Itk to the plasma membrane and hyperresponsiveness to CD3 stimulation. *Mol. Cell. Biol.* **20,** 6945–6957.
27. James, S. Y., Williams, M. A., Newland, A. C., and Colston, K. W. (1999) Leukemia cell differentiation: cellular and molecular interactions of retinoids and vitamin D. *Gen. Pharmacol.* **32,** 143–154.
28. Kohler, G., Baumann, B., Iglesias, A., McCubrey, J., Potash, M. J., Traunecker, A., and Zhu, D. (1984) Different ways to modify monoclonal antibodies. *Med. Oncol. Tumor Pharmacother.* **1,** 227–233.
29. McCubrey, J., McKearn, J. P., and Kohler, G. (1985) Transformation of B and non-B cell lines with the 2,4,6,-trinitrophenyl (TNP)-specific immunoglobulin genes. *Eur. J. Immunol.* **15,** 1117–1124.
30. Dembic, Z., Haas, W., Weiss, S., McCubrey, J., Kiefer H., von Boehmer, H., and Steinmetz, M. (1986) Transfer of specificity by murine alpha and beta T-cell receptor genes. *Nature* **320,** 232–238.
31. Levenson, A. S. and Jordan, V. C. (1997) MCF-7: the first hormone-responsive breast cancer cell line. *Cancer Res.* **57,** 3071–3078.
32. Davis, J. W., Lauer, F. T., Burdick, A. D., Hudson, L. G., and Burchiel, S. W. (2001) Prevention of apoptosis by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in the MCF-10A cell line: correlation with increased transforming growth factor alpha production. *Cancer Res.* **61,** 3314–3320.
33. Budman, D. R., Calabro, A., Wang, L. G., Liu, X. M., Stiel, L., Adams, L. M., and Kreis, W. (2000) Synergism of cytotoxic effects of vinorelbine and paclitaxel in vitro. *Cancer Invest.* **18,** 695–701.
34. Weinstein-Oppenheimer, C. R., Henriquez-Roldan, C. F., Davis, J. M., Navolanic, P. M., Saleh, O. A., Steelman, L. S., et al. (2001) Role of the Raf signal transduction cascade in the *in vitro* resistance to the anticancer drug doxorubicin. *Clin. Cancer Res.* **7,** 2898–2907.
35. Weinstein-Oppenheimer, C. R., Burrows, C., Steelman, L. S., and McCubrey, J. A. (2002) The effects of β-estradiol on Raf activity, cell cycle progression and growth factor synthesis in the MCF-7 breast cancer cell line. *Cancer Biol. Ther.* **1,** 254–260.
36. Davis, J. M., Weistein-Oppenheimer, C. R., Steelman, L. S., Navolanic, P. M., Hu, W., Konopleva, M., et al. (2003) Raf-1 and Bcl-2 induce distinct and common pathways which contribute to breast cancer drug resistance. Submitted to *Clin. Cancer Res.*, in press.

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Elucidation of Signal Transduction Pathways by Transfection of Cells with Modified Oncogenes

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Abstract

This chapter will focus on introduction of various wild type (WT) and mutant genes into cells by DNA transfection. Techniques for analysis of the inheritance, expression, and biological effects of the introduced genes will be described. Various strong and weak points about three different techniques of stable gene transfer, including calcium-phosphate DNA precipitation, transfection via liposomes, and transfection via electroporation, will be discussed.

Key Words: Oncogenes; gene transfer; transfection; liposomes; hematopoietic cells; breast cells; fibroblast cells.

1. Introduction

A preliminary method to investigate function of a particular gene is to introduce the gene into a cell line and determine its effects on growth properties of cells ***(1–9)***. The gene may be wild type (WT) or various other mutant versions, which are either naturally occurring or made by manipulations in the laboratory ***(10–27)***. Various mutant genes include constitutively active (CA) ***(8,9)***, conditionally active (Cond) ***(8,10–14)***, dominant negative (DN), and antisense (AS) ***(19)***. Growth is a fairly broad concept, that can be measured by proliferation, increase in cell size or shape as quantitated by flow cytometric (FACS) or Coulter counting analysis, induction or prevention of apoptosis, loss of contact

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inhibition, loss of requirement for a substratum, growth in soft agar, tumorigenicity, or drug resistance ***(25–29)***. This concept stems from the 1970s and 1980s when many investigators determined whether particular acutely-transforming oncogenic retroviruses would induce morphological transformation of NIH-3T3 or chicken embryo fibroblast (CEF) cells or tumorigenicity upon injection of susceptible mice or chickens ***(30)***. These studies were often performed before the function of a particular oncogene contained in a transforming retrovirus was known. Scientists first focused upon the growth, antigenic properties, and cell lineages of "transformed" cells. Through further investigations scientists discovered biochemical functions of virally encoded oncogenes ***(30)***. Furthermore, these investigators made the seminal discovery that these cancer causing genes were also present in nonmutated evolutionarily conserved forms in genomes of humans and other animals. Initially, many studies were performed to determine how activated oncogenes altered cellular morphology and increased cellular metabolism (e.g., glucose transport) of cells. Studies performed in the 1980s and 1990s determined that oncogenes are involved in signal transduction pathways ***(30)***. Initial studies with retrovirally encoded oncogenes usually consisted of infecting the cell with the naturally occurring oncogenic retrovirus (e.g., Abelson murine leukemia virus [v-*abl*], Rous sarcoma virus [v-*src*], Moloney murine sarcoma virus [v-*mos*]) ***(8,28,30)***. These studies determined the ability of a given oncogene to transform a particular cell line, but were often restricted to studies with either murine or avian cells owing to the host range specificities of the particular virus, although it is noted that some xenotropic and amphitropic retroviruses were made that enabled transfer of these oncogenes into cells from different species. Furthermore, the retrovirally infected cell line was normally selected due to the cells being malignantly transformed as they had altered growth properties (anchorage-independent growth, ability to grow in soft agar, capacity to grow in low concentrations of FBS, ability to grow in the absence of a previously required growth factor [e.g., interleukin 3 (IL-3)], or capability to form a tumor upon injection into immunocompromised mice). A pitfall of this approach was that one could only study malignant transformation with most acutely transforming retroviruses, which had a significant effect on cell growth that enabled the transformed cells to be readily recovered. Some scientists isolated or generated temperature-sensitive (TS) mutant oncogenes, i.e., the oncogene in the retrovirus contained a TS mutation that allowed its activity at the permissive temperature, but not at the restrictive temperature because the mutant TS oncoprotein assumed a conformation which precluded its activity ***(30)***. However, relatively few of these TS oncogenic retroviruses were made and the difference between the restrictive (39.5ºC) and the permissive (35.5ºC) temperature in mammalian cells could be quite small. Moreover, the restrictive temperature was often close to the temperature of induction of heat shock proteins.

The next leap in this field was the concept of dominant selectable markers, which could be incorporated into viral and plasmid gene transfer vectors ***(31, 32)***. Use of these critical tools originated from the field of somatic cell genetics, which used biochemical markers to select for fusion of cells of different types or species. This field had identified certain mutations in genes involved in nucleotide biosynthesis, which rendered cells sensitive to particular drugs. For example, growth of cells having a mutation of the gene encoding hypoxanthine phosphoribosyltransferase (HGPRT), which is necessary for the salvage pathway of purine biosynthesis, was inhibited by blockade of *de novo* purine biosynthesis with aminopterin ***(32,33)***. However, cells transfected with WT *HGPRT* or fused with cells harboring a WT *HGPRT* gene grew in the presence of hypoxanthine, aminopterin, and thymidine (HAT medium) because they had a functional salvage pathway. This later scenario served as a basis for the initial method of producing monoclonal antibodies (Abs). Immortal myeloma (B-cell plasmacytoma) cells with defective HGPRT genes were fused to normal mortal splenocytes harboring rearranged Ig and WT HGPRT genes. Only fused cells containing the myeloma gene(s) for immortalization and the WT splenocyte HGPRT genes would grow in HAT medium. Monoclonal Abs were later derived following screening of the clones with the appropriate antigen and the clone expressing the desired antibody was identified and amplified ***(33)***.

Initial somatic cell genetic studies were limited to cells that had preexisting mutations in genes such as HGPRT or thymidine kinase (TK). However it was soon shown that there was a bacterial gene, xanthine-guanine phosphoribosyl transferase (*gpt*), which would substitute for mammalian HGPRT ***(32)***. $HGPRT^{-/-}$ cells transfected with the bacterial *gpt* grew in HAT medium ***(32)***, and importantly, it was shown that *gpt* conferred resistance to the antibiotic mycophenolic acid ***(33)***. This was a critical discovery because mammalian cells are sensitive to this antibiotic. When normal mammalian cells are cultured in medium containing mycophenolic acid, they die unless they contain the bacterial *gpt* gene. If a "test" gene is inserted into the vector, that contains the *gpt* gene, it is possible to introduce the "test" gene into the cells and select for stable transformants by culturing the cells in the presence of mycophenolic acid. This "test" gene was regulated by SV40 enhancer sequences present in the vector (pSV2gpt). This was the important start of a new exciting era of gene transfection as it was now possible to modify the desired genes and insert them into a vector with a dominant selectable marker, transfect them into various types of cells and determine their effects on cell growth, signal transduction, apoptosis, etc. A variety of bacterial antibiotic resistance genes were identified that would function in mammalian cells. These resistance genes included neo^r, encoding resistance to the antibiotic G418 (geneticin), $hygro^r$ encoding resistance to the antibiotic hygromycin, $puro^r$ encoding resistance to the antibiotic puromycin, and $blast^r$

encoding resistance to the antibiotic blasticiden. Some of the initial vectors, which were commonly used, were developed by Mulligan and Berg and called pSV2neo, pSV2gpt, pSV2hygro ***(32)***.

It is possible to transfect a cell with a gene in one vector encoding a particular antibiotic resistance marker and then after stable transfectants are isolated, another gene in a different vector containing a different antibiotic resistance marker could be introduced into the cells and so on. Although it is theoretically possible to introduce two different vectors into cells at once, and perform a double selection, this may not in practice work very effectively due to the low efficiency of stable gene transfer.

A drawback of these initial studies was that the original vectors were usually plasmids as opposed to retroviral vectors. It is possible to efficiently introduce genes into adherent fibroblastic cells by calcium phosphate or synthetic liposomes but these techniques are not that effective for introducing genes into hematopoietic cells (An outline of stable gene transfer into fibroblast cells is presented in **Fig. 1**). Other techniques such as electroporation (*see* **Fig. 2**) and the less commonly used bacterial protoplast fusion can introduce genes into hematopoietic cells, however, the efficiency of recovery of stable transfected cells can be quite low (10^{-6} to 10^{-7}) and also vary between different laboratories substantially.

2. Materials

2.1. Calcium Phosphate Mediated Gene Transfer

1. DNA encoding the gene of interest, usually in the form of plasmid DNA.
2. Bacteria harboring plasmid DNA.
3. LB bacterial broth.
4. LB agar Petri dishes containing appropriate antibiotics (*see* **Note 1**).
5. Restriction endonucleases (REs) [various biotech companies including Invitrogen (San Diego, CA), Promega (Madison, WI), Roche Diagnostics (Indianapolis, IN), and New England Biolabs (Beverly, MA)] to verify that insert DNA is properly oriented.
6. 5X or 2X HEPES buffer: 1X HEPES buffer is 140 m*M* NaCl, 5 m*M* KCl, 4 m*M* Na_2HPO_4, 10 m*M* dextrose, 20 m*M* HEPES, final pH 7.05 (*see* **Note 1**).
7. Sterile 1.25 *M* $CaCl_2$ stock solution.
8. DMSO.
9. Sucrose.
10. Dulbecco's modified Eagle's Medium (DMEM, Invitrogen).
11. Fetal bovine serum (FBS) [Atlanta Biologicals (Atlanta, GA), Hyclone, (Logan, UT), or (Invitrogen)] or iron supplemented defined bovine calf serum (BCS, Hyclone).
12. Phosphate buffered saline (PBS).
13. Antibiotics: blasticiden (Invitrogen), geneticin (G418) (Invitrogen), hygromycin (Calbiochem, San Diego, CA), and puromycin (Sigma).
14. 60 mm tissue culture plates (Corning, Corning, NY).
15. T25 and T75 tissue culture flasks (Corning).

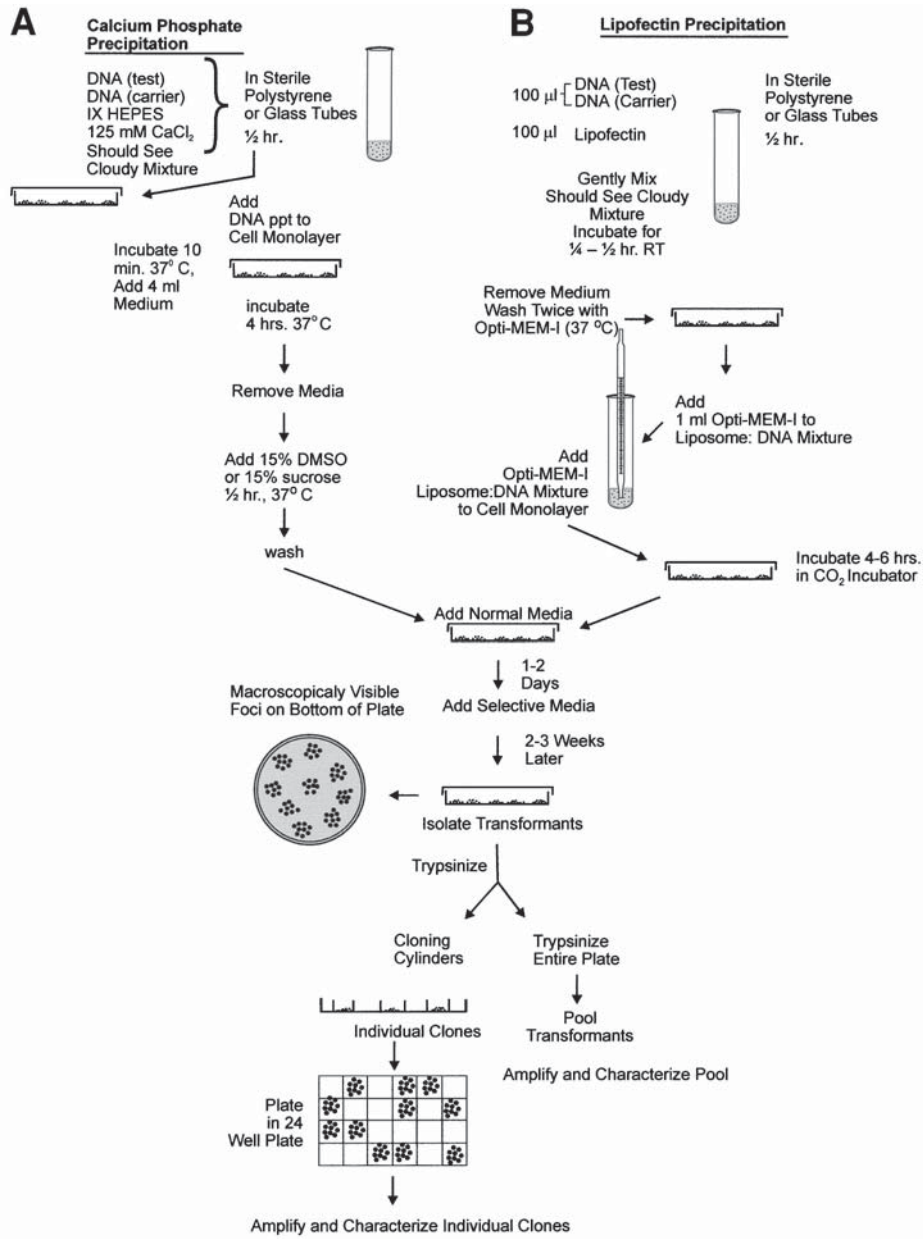
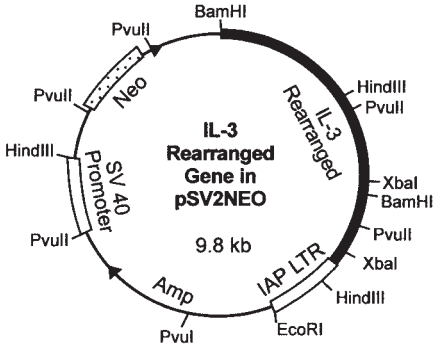


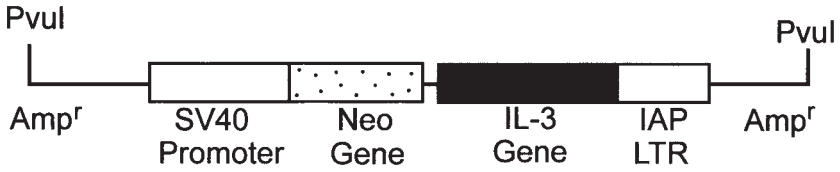
Fig. 1. Outline of stable gene transfer methods for fibroblast cells. An outline of calcium precipitation and liposome mediated gene transfer is presented. These techniques are effective for transfection of adherent cells.

A

1. Grow plasmid DNA containing gene of interest and isolate plasmid DNA.



2. Linearize Plasmid DNA which cuts once in a nonessential region for selection in eukaryotic cells.



3. Check on agarose gel to determine that DNA is cleaved to yield one band.

4. Grow hematopoietic cells.

5. Deprive hematopoietic cells of cytokine for 24 hr to synchronize cells in G_0/G_1 cell cycle phase.

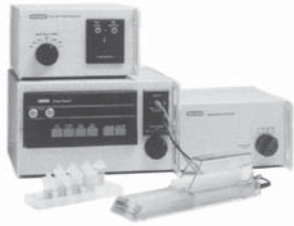
6. Incubate cell in IL-3 for 24 hr to enrich for cells in S phase.

7. Resuspend 10 to 20 μg of linearized DNA with 1 x 10^7 cells in 500 μl a BioRad electroporation cuvet.

Fig. 2. Outline of stable plasmid transfer into hematopoietic cells. The technique of electroporation of DNA contained in eukaryotic expression vectors containing a dominant selectable marker is illustrated.

8. Place electroporation cuvet with cells on ice for 10 minutes.

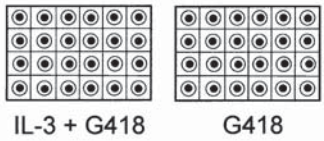
9. Electroporate cells using a BioRad Gene Pulser at 350V and 950 μFarads.



10. Incubate cells in 100 mm petri dishes with 20% FBS and 20% WEHI-3B supernatant (cytokine source) for 2 days.

11. Collect cells by pipeting cells off dish.

12. Plate in 96 round bottom well plates in selective conditions with multichannel pipettor.

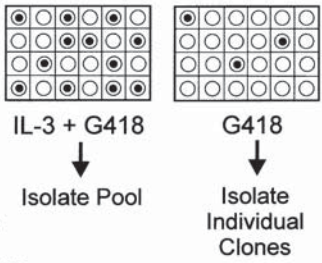


13. Feed every three days.

14. Every second feeding, gently remove 150 μl of medium, then add 100 μl of fresh medium.

Only Stable Transformants Grow

15. After 2 weeks, score electroporation results. Look at bottom of plate for foci of cells growing in a mound or medium becomes yellow.



16. Characterize growth properties, DNA copy number, expression of transgene, effects on signal transduction and apoptotic pathways, drug resistance, and tumorigenicity in nude mice.

Fig. 2. (*continued*)

2.2. Liposome-Mediated DNA Transfection

1. Lipofectin® (Invitrogen).
2. Opti-MEM-I medium (Invitrogen).
3. Sterile 12 × 75 mm polystyrene or glass tubes (Fisher, Raleigh, NC).

4. Sterile disposable glass pipets.
5. DMEM.
6. PBS.
7. Antibiotics: blasticiden, geneticin (G418), hygromycin, and puromycin.
8. 60-mm tissue culture plates.
9. T25 and T75 tissue culture flasks (Corning, Corning, NY).
10. Hollow glass rods or cloning cylinders.
11. 1X Trypsin solution.
12. Clean Vaseline or silicone grease.

2.3. DNA Transfection by Electroporation

1. Bacteria.
2. Eukaryotic gene transfer plasmid containing a dominant selectable marker.
3. REs.
4. Agarose.
5. Horizontal electrophoresis apparatus (e.g., 10×7 cm) (various sources including Invitrogen and BioRad, Hercules, CA).
6. BioRad Electroporation Gene Pulser (Bio Rad).
7. Electroporation cuvettes (BioRad).
8. PBS.
9. 100-mm Petri dishes.
10. 96-well round bottom plates.
11. Ice bucket and ice.
12. Eukaryotic growth medium.
13. Antibiotics: blasticiden, geneticin (G418), hygromycin, and puromycin.

3. Methods

3.1. Calcium-Phosphate-Mediated Gene Transfer

This technique works well with fibroblast cells and is inexpensive ***(34)***. If one is going to perform numerous transfections at once with fibroblasts, it is less expensive than using commercially available liposomes (*see* **Fig. 1**).

1. Grow eukaryotic expression vector in bacteria, isolate plasmid DNA.
2. Insert "test" gene of interest into multiple cloning site of an eukaryotic expression vector.
3. Ensure insert is in correct orientation by RE digestion and mapping or DNA sequencing (go to **step 6**).
4. If you are not going to subclone the "test" DNA into eukaryotic expression vector harboring a dominant selectable marker, obtain "test" gene of interest in the form of plasmid DNA, grow a large culture of bacteria harboring the plasmid DNA, then perform plasmid DNA isolation. Also, amplify a bacterial culture harboring

a plasmid DNA encoding the appropriate selectable marker gene for selection in eukaryotic cells (e.g., *neo*^{r}, *gpt*^{r}, *hygro*^{r}, *blast*^{r}, or *puro*^{r}).

5. A mixture containing the "test" gene of interest (nonselectable DNA, 10-50 μg) is mixed with the dominant selectable marker DNA (1 μg) and the final DNA amount is adjusted to 10–50 μg by the addition of carrier DNA that does not have DNA sequences homologous to the gene of interest or the dominant selectable marker (usually, sheered denatured calf thymus DNA). The above DNA quantities are based upon one transformation experiment. The carrier DNA is used in two additional cases, first as a control without your "test" DNA (just dominant selectable marker) and second as a control without your dominant selectable marker (mock control). If you want to examine DNA from a patient or a human cell line for a potential oncogene, you can use genomic DNA prepared from these sources and omit the carrier DNA. This procedure is often done to determine if DNA from a patient or cell line has a mutant oncogene which will transform NIH-3T3 cells. DNA is diluted with either 5X or 2X HEPES buffer to a final concentration of 1X HEPES buffer. The DNA mixture is adjusted to 125 m*M* $CaCl_2$, usually with one-tenth volume of 1.25 *M* $CaCl_2$ stock solution. A precipitate is allowed to form for 30 min at room temperature.
6. Remove medium from cells (usually fibroblast), which were seeded 1 d earlier at 2×10^5 to 5×10^5 cells/60 mm tissue culture plate. Cells should be 50% confluent. Cells should be seeded in a medium such as Iscoves modified Dulbecco's medium (IMDM), Dulbecco's modified Eagle medium (DMEM), or minimal essential medium (MEM) (*see* **Note 2**).
7. Gently add the DNA precipitated with calcium phosphate to the plate in a dropwise fashion. Rock the plate gently to evenly distribute the precipitate, before placing in the 37ºC, 5% CO_2 incubator. Incubate for 10 minutes.
8. At the end of the incubation gently add 4 mL of complete medium and then incubate for 4 h and then remove media by aspiration and add fresh medium, then go to **step 10**.
9. Alternatively, at the end of the four hour incubation, add prewarmed 15% DMSO (in medium) or 15% sucrose (in medium) to the plates, then incubate for 30 min in the 37ºC, 5% CO_2 incubator. These are DMSO and sucrose shock treatments. Cells must be washed with media after shock treatments. Shock treatments result in an approximately two- to fivefold increase in transformation frequency of fibroblast cells.
10. One to two days later, appropriate selective medium is added to the cells (depending upon which dominant marker is utilized, *see* **Table 1**). One must titer the amount of antibiotic necessary to kill all mock-transfected cells. Keep cells in culture with repeated changes of medium containing the antibiotic to ensure all mock-transfected cells are dead. Foci of transfected cells are usually macroscopically visible 8–10 d posttransfection by carefully holding the plate up and examining at the bottom of the plate for a round speck. Foci can be confirmed with a microscope. Fibroblastic transformants should be isolated when sufficient cells (100–1000 cells/

Table 1
Drug Concentrations for Selection of Stably Infected Cells

	Type of Cells		
Antibiotic	Muine Fibroblast	Murine Hematopoietic	Human Hematopoietic
G418	500 μg/mL to 1000 μg/mL	2 mg/mL	2 mg/mL
Puromycin	4 μg/mL	1 to 2 μg/mL	1 to 2 μg/mL
Blasticiden	25 μg/mL	25 to 50 μg/mL	25 to 50 μg/mL
Hygromycin	200 μg/mL	400 μg/mL	400 μg/mL

foci) are available with cloning cylinders (2 × 1 cm sections of a hollow glass rod). Alternatively, transformants can be grown into bulk cultures and screened for the desired phenotype (*see* **Note 3**).

3.2. Liposome-Mediated Gene Transfer

1. Purchase Lipofectin® or similar product. Store Lipofectin® at 4ºC because storage at room temperature will decrease transfection effectiveness. Record date that Lipofectin® arrives so that the age of the product is known. Lipofectin® is stable for up to 2 yr if kept refrigerated.
2. Seed appropriate adherent cell line in 60 mm plates at 2×10^5 to 5×10^5 cells/plate (approx 50–75% confluent) 1 d in advance.
3. Obtain plasmid DNA as described in **Subheading 3.1., steps 1–3.**
4. Determine the concentration of DNA, then aliquot 25–50 μg DNA in sterile polystyrene tubes (12 × 75 mm). Titrate the amount of DNA optimal for the cell line, because some cell lines are sensitive to the relatively large amounts of DNA indicated above. However, if insufficient DNA is used, optimal levels of liposome formation and DNA transfer will not be obtained (*see* **Note 4**).
5. Adjust DNA volume to 100 μL with sterile H_2O.
6. Include a negative mock control (e.g., no plasmid DNA containing antibiotic resistance marker) to ensure that the antibiotic concentration kills off all non-transfected cells.
7. Remove 100 μL of Lipofectin® (equal volume as DNA solution) with sterile glass disposable 1 mL pipet, add gently to tube containing 100 μL DNA, then mix gently. This should present a cloudy solution. This is the DNA:liposome complex. If one does not see this cloudy solution, it may indicate the DNA concentration is too low and should be increased.
8. Incubate the DNA:liposome mixture for 15–30 min at room temperature in a sterile tissue culture hood. In the meantime, warm a bottle of Opti-MEM-I medium (Invitrogen) to 37ºC.
9. At the end of the room temperature incubation, remove medium from cultured fibroblasts by aspiration with a sterile Pasteur pipette. Wash cells twice with 2 mL

of Opti-MEM-I medium. Finally, remove Opti-MEM-I medium from the plate. Opti-MEM-I has low serum and lacks antibiotics.

10. Gently add 1 mL of Opti-MEM-I medium to the DNA:Lipofectin® mixture by dribbling Opti-MEM-I down the tube. Gently resuspend the mixture with a pipette.
11. Add the Opti-MEM-I:DNA:liposome mixture dropwise to the Petri dish containing cells after removal of the tissue culture medium.
12. Gently rock the plate to spread the Opti-MEM-I:DNA:liposome mixture over the plate then place it into a CO_2 incubator for 4–6 h. At the end of the incubation, add 3 mL of normal medium containing serum to the plate. Incubate for 2 d.
13. Add normal medium containing serum and selective antibiotic (e.g., G418, puromycin, hygromycin, or blasticiden), then change medium every three days after aspiration of the old medium from the plate.
14. After 12 d of incubation, foci of cells on the bottom of the plate, which represent transfected cells, should be visible macroscopically, which can be confirmed microscopically. At this point, one can either isolate clones with glass cloning cylinders or isolate pools of transfected cells. Pools of 100–200 foci (with 100–1000 cells/foci) are usually preferred, as they will yield more representative results. However, you may want both clones and pools.
15. Glass-cloning cylinders are small (1 cm) hollow glass rods that should be etched with a file, broken at about 1 cm of length, autoclaved in a glass Petri dish. When one is ready to start isolation of clones, the bottom of the cloning cylinder is gently dipped in a jar of clean vaseline petroleum jelly or silicon grease with sterile forceps. This allows the glass cylinder to adhere to the Petri dish over foci. A little pressure is applied to seal the cloning cylinder to the Petri dish over the focus that will be collected. Usually, if Vaseline or silicone grease is clean and only used for this purpose one should have not problems with sterility. Cells on the plate in the cloning cylinder are briefly washed by pipeting a few drops of sterile 1X trypsin with a Pasteur pipet into the cloning cylinder. Do not let the trypsin leak out from the cloning cylinder (this is why a seal is created with the cloning cylinder using Vaseline or silicone grease), otherwise, you will not obtain clones if there are many individual foci on the dish. After 10 min of incubation, cells are recovered in trypsin by gently pipeting up and down with a Pasteur pipet and transferring to one well of a 24-well tissue culture plate in 1 mL of normal medium. Multiple clones should be isolated because not every attempt at recovering cells from cloning cylinders will be successful. One can further subclone these clones by performing limiting dilution analysis by plating cells at approx 0.1 cells/well in 96-well plates, then isolating the clones that arise in different wells. One should recover approx 10 clones by this process if the cells have a plating efficiency of one in your culture conditions.

3.3. Electroporation-Mediated Gene Transfer (see Fig. 2)

1. Grow bacteria harboring plasmid containing gene of interest and dominant selectable marker (*see* **Note 5**). Isolate plasmid DNA as described in **Subheading 3.1., steps 1–3.**

2. Two days prior to transfection cytokine deprive cells to synchronize them in the G_0/G_1 cell-cycle phase.
3. After one day of starvation add IL-3 (or the growth factor required for growth) to cells so they re-enter the cell cycle, are exponentially growing, and contain a high percentage of the cells in S phase.
4. Linearize the plasmid DNA with a RE that cuts in a nonessential position of the plasmid. In the case shown in **Fig. 2** that deals with introducing a rearranged IL-3 gene into nonadherent hematopoietic cells, one can cleave with an RE (Pvu I), which cuts in the *amp*^{r} gene, because it is no longer necessary. Electrophorese DNA to ensure that the plasmid has been linearized.
5. Resuspend 10 to 20 µg of linearized plasmid DNA with 1×10^7 cells in 500 µL in a BioRad Electroporation Gene Pulser Cuvette (BioRad). Put cuvette containing cells and DNA on ice for 10 min.
6. Electroporate cells using a BioRad Gene Pulser at 305 V and 950 µFarads (*see* **Note 6**).
7. Plate cells in 100-mm Petri dishes with IMDM + 20% FCS + 20% WCM (source of IL-3). Incubate cells for 2 d.
8. Collect cells by pipeting (make sure that you pipet the cells up and down on the plate as even though they are nonadherent, many nonadherent cell lines loosely stick to the plates). Pellet cells by centrifugation (500 to 1000*g* for 5 to 10 min), resuspend cells in sterile PBS, collect cells again by centrifugation, and then plate in selective conditions in 96-well plates. An outline of this technique is shown in **Fig. 2**.
9. Determine whether cells have inherited DNA of interest by Southern blot analysis. Determine that cells express the gene by RNA and protein analysis. Determine whether gene affects cellular growth properties (*see* **Note 7**).

4. Notes

1. Antibiotics commonly used include ampicillin (stock solution is 25 mg/mL and working solution 35–50 µg/mL) (Sigma, St. Louis, MO) and tetracycline (stock solution is 12.5 mg/mL and working solution is 12.5–15 µg/mL) (Sigma). Add Ab to autoclaved agar when it has cooled to approx 42º–45ºC, otherwise the heat will inactivate the Ab. The molten agar containing antibiotics is mixed by swirling, then poured into Petri dishes. HEPES buffer should be sterilized by filtration as opposed to autoclaving because it contains dextrose.
2. In some media (e.g., McCoy's 5A), the media will be insoluble when the calcium phosphate DNA precipitate is added due to extra salts in the media and, thus, these media should not be used.
3. The calcium phosphate precipitation technique is useful for stably transfecting fibroblast cells, but hematopoietic cells are not susceptible to transfection.
4. DNA should be dissolved in 1X Tris-EDTA (TE, pH 7.6) or sterile H_2O, and not in buffer with $NaCl_2$ (e.g., TEN).

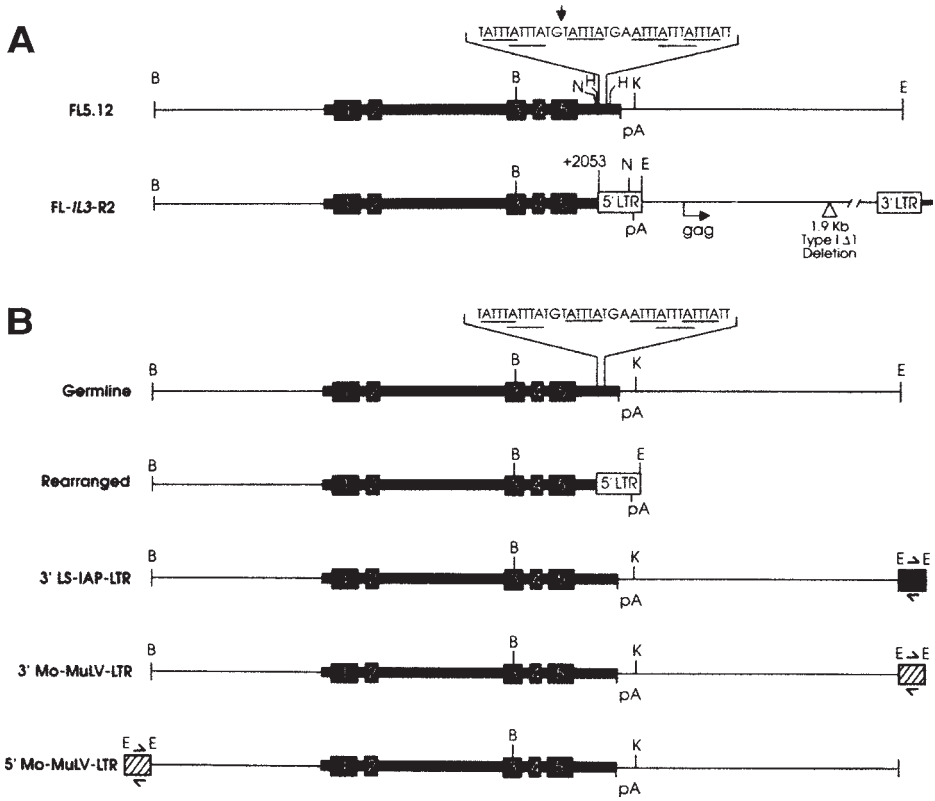


Fig. 3. Recombinant IL-3 constructs used to elucidate effects of IL-3 gene rearrangement on IL-3 expression. Illustration of modified IL-3 genes used to determine the mechanisms of hematopoietic cell transformation induced by the rearranged IL-3 gene. Germline (gIL-3) and rearranged (rIL-3) genes were cloned from a cell line determined to have a rearranged IL-3 gene (FL-IL3-R) (15-17). To determine the mechanism by which rearrangement altered expression of IL-3, portions of the rIL-3 gene were removed and replaced by other LTR sequences present in different retroviruses (e.g., IAP, Mo-MuLV) and recombinant IL-3 genes were inserted into pSV2neo. These constructs were electroporated into IL-3 dependent cells as described in Fig. 2 and inheritance and effects on gene expression were determined as described in Fig. 4.

5. In order to determine how a genetic mutation at the IL-3 gene resulted in the autocrine transformation of the IL-3 gene ***(15)***, we cloned the germline and rearranged (mutant) IL-3 gene from the cells harboring the rearranged IL-3 gene. We are discussing these studies as they represent an example to understand how gene transfer by electroporation can be used to examine the effects of gene mutation in nonadherent cells. Various deletion and additions were introduced into rearranged

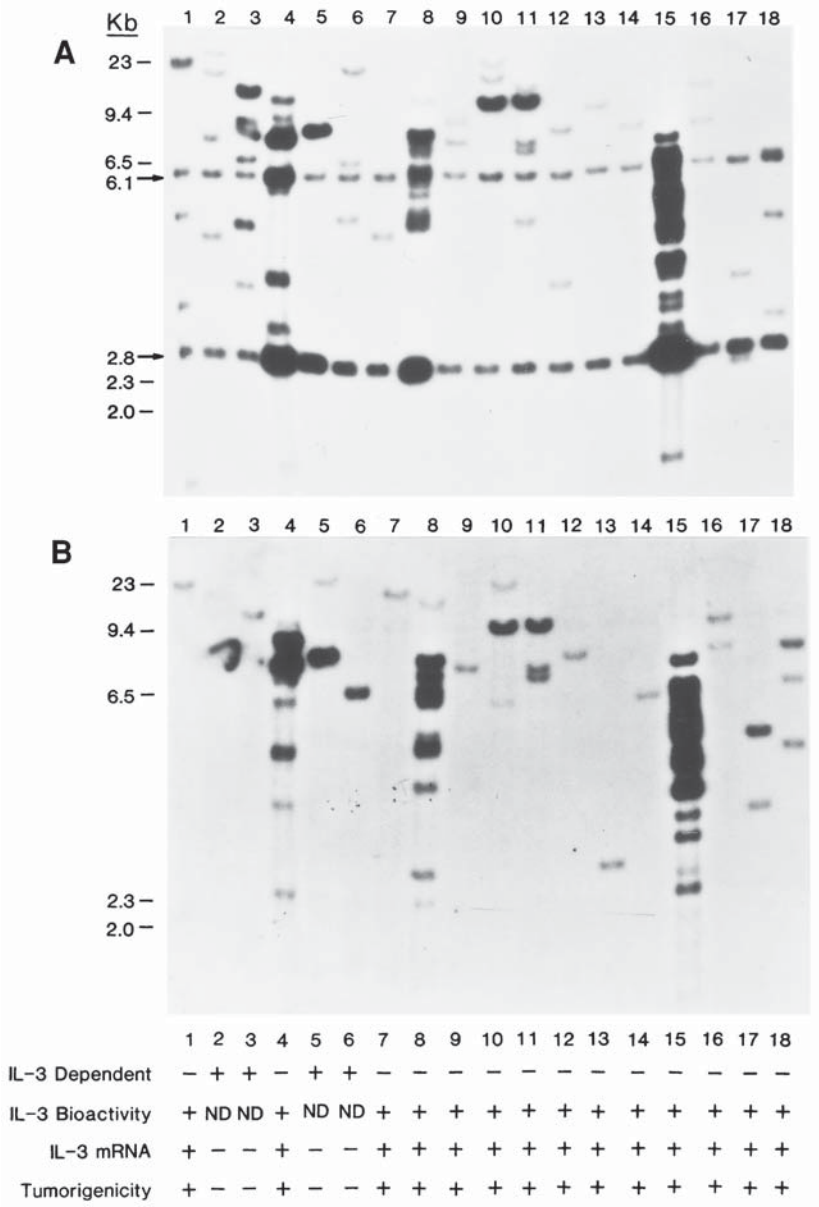


Fig. 4. Characterization of inheritance of introduced IL-3 gene in hematopoietic cells. Inheritance of rIL3 and gIL-3 gene in electroporated FL5.12 cells. BamHI digested DNA was hybridized with IL-3 and neo probes in Panels (**A**) and (**B**), respectively. The same filter was used in both hybridizations. The bands at 6.1 Kb and 2.8 Kb in Panel A represent the endogenous IL-3 gene fragments. Note these endogenous IL-3 gene fragments are not detected in Panel B with the *neo* probe. Additional bands in

and germline IL-3 genes by standard cloning techniques and subcloned into pSV2neo (*see* **Fig. 3**). Plasmid DNA was electroporated into IL-3-dependent FL5.12 cells as described in **Fig. 2**. Effects of different constructs on isolation of cytokine-independent cells was determined by plating transfected cells in medium containing G418 (to select for inheritance of various pSV2neo plasmids) in the presence and absence of exogenous IL-3.

6. One may see a lot of dead cells in the culture after electroporation of hematopoietic cells because electroporation is a harsh treatment. We have observed transformation frequencies of $<10^{-6}$ to 10^{-8} with three different hematopoietic cell lines (FL5.12, FDC-P1, and TF-1 cells).
7. It is important to determine that the transfected cells have actually inherited the plasmid DNAs. This was determined by Southern blot analysis of DNAs isolated from different cell lines (*see* **Fig. 4**). Note that some transfected cells had inherited multiple transgene copies. This is typical of gene transfer experiments by calcium phosphate precipitation and electroporation. Moreover, higher levels of transgene expression are usually associated with higher DNA copy numbers ***(15–17)***. Effects of the introduced IL-3 gene on IL-3 mRNA, bioactivity, and tumorigenicity is shown.

References

1. Blalock, W. L., Weinstein-Oppenheimer, C., Chang, F., Hoyle, P. E., Wang, X. Y., Algate, P. A., et al. (1999) Signal transduction, cell cycle regulatory, and anti-apoptotic pathways regulated by IL-3 in hematopoietic cells: possible sites for intervention with anti-neoplastic drugs. *Leukemia* **13,** 1109–1166.
2. McCubrey, J. A., May, W. S., Duronio, V., and Mufson, A. (2000) Serine/threonine phosphorylation in cytokine signal transduction. *Leukemia* **14,** 9–21.
3. Wang, X. Y., Steelman, L. S., and McCubrey, J. A. (1997) Abnormal activation of cytokine gene expression by intracisternal type A particle transposition: effects of mutations which result in autocrine growth factor stimulation and malignant transformation. *Cytokines Cell Mol. Ther.* **3,** 3–19.

Fig. 4. (*continued*). Panel A represents DNA fragments which hybridize with the IL-3 probes. Note that some clonal cell lines have inherited multiple copies of the IL-3 gene (Lanes 4, 8, 15). DNA prepared from: Lane 1) FL-rIL3-T1, 2) FL-rIL3-T2, 3) FL-gIL-3 + 3' MoMuLV-LTR-T1, 4) FL-gIL-3 + 3' MoMuLV-LTR-T2, 5) FL-gIL-3 + 3' MoMuLV-LTR-T3', 6) FL-gIL-3 + 3' MoMuLV-LTR-T4, 7) FL-gIL-3 + 5' MoMuLV-LTR-T1, 8) FL-gIL-3 + 5' MoMuLV-LTR-T2, 9) FL-gIL-3 + 5' MoMuLV-LTR-T3, 10) Fl-gIL-3 + 5' MoMuLV-LTR-T4, 11) FL-gIL-3 + 3' IAP-LTR-T1, 12) FL-gIL-3 + 3' IAP-LTR-T2, 13) FL-gIL-3 + 3' IAP-LTR-T3, 14) FL-gIL-3 + 3' IAP-LTR-T4, 15) FL-gIL-3 + 3' IAP-LTR-T5, 16) FL-gIL-3 + 3' IAP-LTR-T6, 17) FL-gIL-3 + 3' IAP-LTR-T7, and 18) FL-gIL-3 + 3' IAP-LTR-T8. Cytokine dependence, presence of IL-3 mRNA and protein and ability of cells to form tumors in immunocompromised mice is indicated under Panel **B**.

4. White, M. K. and McCubrey, J. A. (2001) Suppression of apoptosis: role in cell growth and neoplasia. *Leukemia* **15,** 1011–1121.
5. Lee, J. T. Jr. and McCubrey J. A. (2002) The Raf/MEK/ERK signal transduction cascade as a target for chemotherapeutic intervention in leukemia. *Leukemia* **16,** in press.
6. Weinstein-Oppenheimer, C. R., Blalock, W. L, Steelman, L. S., Chang, F., and McCubrey, J. A. (2000) Raf signal transduction cascade as a target for chemotherapeutic intervention in growth factor-dependent tumors *Pharmacol. Ther.* **88,** 229–279.
7. McCubrey, J. A., Lee, J. T., Steelman, L. S., Blalock, W. L., Moye, P. W., Chang, F., et al. (2001) Interactions between the PI3K and Raf signaling pathways can result in the transformation of hematopoietic cells. *Cancer Detect. Prev.* **25,** 375–393.
8. McCubrey, J. A., Smith, S. R., Algate, P. A., DeVente, J. E., White, M. K., and Steelman, L. S. (1993) Retroviral infection can abrogate the factor-dependency of hematopoietic cells by autocrine and non-autocrine mechanisms depending on the presence of a functional viral oncogene. *Oncogene* **8,** 2905–2915.
9. McCubrey, J., Steelman, L. S., Wang, X.-Y., Davidian, E. W., Hoyle, P. E., White, C., et al. (1995) Autocrine growth factor secretion after transformation of human cytokine-dependent cells by viral and cellular oncogenes. *Int. J. Oncol.* **7,** 573–586.
10. Hoyle, P. E., Moye, P. W., Steelman, L. S., Blalock, W. L., Franklin, R. A., Pearce, M., et al. (2000) Differential abilities of the Raf family of protein kinases to abrogate cytokine-dependency and prevent apoptosis in murine hematopoietic cells by a MEK1-dependent mechanism. *Leukemia* **14,** 642–656.
11. Moye, P. W., Blalock, W. L., Hoyle, P. E., Chang, F., Franklin, R. A., Weinstein-Oppenheimer, C., et al. (2000) Synergy between Raf and BCL2 in abrogating the cytokine dependency of hematopoietic cells. *Leukemia* **14,** 1060–1079.
12. Blalock, W. L., Moye, P. W., Chang, F., Pearce, M., Steelman, L. S., McMahon, M., and McCubrey, J. A. (2000) Combined effects of aberrant MEK1 activity and BCL2 overexpression on relieving the cytokine-dependency of human and murine hematopoietic cells. *Leukemia* **14,** 1080–1096.
13. Blalock, W. L., Moye, P. W., Steelman, L. S., Franklin, R. A., McCarthy, S. A., Cherwinski, H., et al. (2000) A conditionally-active form of MEK1 results in autocrine transformation of human and mouse hematopoietic cells. *Oncogene* **19,** 526–536.
14. Blalock, W. L., Moye, P. W., Chang, F., Lee, J. T., Pohnert, S. C., Burrows, C., et al. (2001) Effects of inducible MEK1 activation on the cytokine dependency of lymphoid cells. *Leukemia* **15,** 794–807.
15. Mayo, M., Wang, X.-Y., Algate, P. A., Arana, G. F., Hoyle, P. E., Steelman, L. S., and McCubrey, J. A. (1995) Synergy between AUUUA motif disruption and enhancer insertion result in autocrine transformation of interleukin-3 dependent hematopoietic cells. *Blood* **86,** 3139–3150.
16. Wang, X. Y. and McCubrey, J. A. (1996) Malignant transformation induced by cytokine genes: a comparison of the abilities of germline and mutated interleukin 3 genes to transform hematopoietic cells by transcriptional and posttranscriptional mechanisms. *Cell Growth Differ.* **7,** 487–500.

17. Wang, X. Y. and McCubrey, J. A. (1997) Differential effects of retroviral long terminal repeats on interleukin-3 gene expression and autocrine transformation. *Leukemia* **11,** 1711–1725.
18. McCubrey, J. A., Steelman, L. S., Wang, X.-Y., Algate, P. A., Hoyle, P. E., White, C., et al. (1995) Differential effects of viral and cellular oncogenes on the growth factor-dependency of hematopoietic cells. *Int. J. Oncol.* **7,** 295–310.
19. McCubrey, J. A., Steelman, L. S., Hoyle, P. E., Blalock, W. L., Weinstein-Oppenheimer, C., Franklin, R. A., et al. (1998) Differential abilities of activated Raf oncoproteins to abrogate cytokine-dependency, prevent apoptosis and induce autocrine growth factor synthesis in human hematopoietic cells. *Leukemia* **12,** 1903–1929.
20. Weinstein-Oppenheimer, C., Steelman, L. S., Algate, P. A., Blalock, W. L., Burrows, C., Hoyle, P. E., et al. (2000) Effects of deregulated Raf activation on integrin, cytokine-receptor expression and the induction of apoptosis in hematopoietic cells. *Leukemia* **14,** 1921–1938.
21. McCubrey, J. A., Blalock, W. L., Chang, F., Steelman, L. S., Pohnert, S. C., Navolanic, P. M., et al. (2001) Signal transduction pathways: cytokine model. In: *Cell Cycle Checkpoints and Cancer*, *Molecular Biology Intelligence Unit 15*, (Blagosklonny, M. V., ed.), Landes Bioscience, Austin, TX, pp. 17–51.
22. Hoyle, P. E., Steelman, L. S., and McCubrey, J. A. (1997) Autocrine transformation of human hematopoietic cells after transfection with an activated granulocyte/macrophage colony stimulating factor gene. *Cytokines Cell Mol. Ther.* **3,** 159–168.
23. McCubrey, J., McKearn, J. P., and Kohler, G. (1985) Transformation of B and non-B cell lines with the 2,4,6,-trinitrophenyl (TNP)-specific immunoglobulin genes. *Eur. J. Immunol.* **15,** 1117–1124.
24. Dembic, Z., Haas, W., Weiss, S., McCubrey, J., Kiefer, H., von Boehmer, H., and Steinmetz, M. (1986) Transfer of specificity by murine alpha and beta T-cell receptor genes. *Nature* **320,** 232–238.
25. Weinstein-Oppenheimer, C. R., Henriquez-Roldan, C. F., Davis, J. M., Navolanic, P. M., Saleh, O. A., Steelman, L. S., et al. (2001) Role of the Raf signal transduction cascade in the *in vitro* resistance to the anticancer drug doxorubicin. *Clin. Cancer Res.* **7,** 2898–2907.
26. Weinstein-Oppenheimer, C. R., Burrows, C., Steelman, L. S., and McCubrey, J. A. (2002) The effects of β-estradiol on Raf activity, cell cycle progression and growth factor synthesis in the MCF-7 breast cancer cell line. *Cancer Biol. Ther.* **1,** 254–260.
27. Davis, J. M., Weinstein-Oppenheimer, C. R., Steelman, L. S., Navolanic, P. M., Hu, W., Konopleva, M., et al. (2003) Raf-1 and Bcl-2 induce distinct and common pathways which contribute to breast cancer drug resistance. *Clin. Cancer Res.*, in press.
28. McCubrey, J. A., Holland, G., McKearn, J., and Risser, R. (1989) Abrogation of growth factor dependency in 2 IL-3 dependent cell lines occurs by 2 distinct mechanisms. *Oncogene Res.* **4,** 97–109.
29. McCubrey, J. A., Steelman, L. S., Mayo, M. W., Algate, P. A., Dellow, R. A., and Kaleko, M. (1991) Growth promoting effects of insulin-like growth factor-1 on

hematopoietic cells: overexpression of introduced IGF-1 receptor abrogates IL-3-dependency of murine factor dependent cells by a ligand-dependent mechanism. *Blood* **78,** 921–929.
30. Weiss, R., Teich, N., Varmus, H., and Coffin, J. (1984) RNA tumor viruses. Cold Spring Harbor Press. 2nd Edition.
31. Miller, A. D. and Rosman, G. J. (1989) Improved retroviral vectors for gene transfer and expression. *Biotechniques* **7,** 980–990.
32. Mulligan, R. C. and Berg, P. (1981) Selection for animal cells that express the Escherichia coli gene encoding for xanthine-guanine phosphoribosyltransferase. *Proc. Natl. Acad. Sci. USA* **78,** 2072–2076.
33. Kohler, G. and Milstein, C. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **7,** 495–497.
34. McCubrey, J. (1985) DNA-mediated transformation of lymphoid cells. In: *Immunological Methods.* Vol. III, Orlando, FL, Academic, pp. 21–29.

17

Elucidation of Signal Transduction Pathways by Retroviral Infection of Cells with Modified Oncogenes

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Abstract

This chapter will focus on understanding how various wild type (WT), dominant negative (DN), constitutively active (CA), and conditionally active (COND) oncogenes, as well as antisense (AS) genes contained in retroviral vectors may be used to elucidate signal transduction pathways. We will describe methods to introduce these genes into cells and subsequent analysis of inheritance, expression, and biological effects of the genes introduced. Furthermore, we will discuss various strong points about each of these different types of constructs, how they can be used to elucidate signal transduction, apoptotic, and drug resistance pathways as well as various pitfalls commonly encountered with their usage.

Key Words: Retroviral vectors; oncogenes; gene transfer; conditional constructs; Raf; MEK; PI3K; Akt; protein kinases; signal transduction inhibitors; drug resistance; hematopoietic cells; breast cells; fibroblast cells.

1. Introduction

In the early 1980s, the construction of recombinant retroviral vectors revolutionized the fields of gene transfer and therapy ***(1–3)***. Recombinant retroviral vectors enabled insertion of viral or cellular oncogenes (usually less than 5 kb)

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into a retroviral backbone that lacked many genes necessary for retroviral replication but contained retroviral transcription regulatory long terminal repeat (LTR) and packaging sequences (Psi2, Ψ2) necessary for packaging the recombinant retroviral genome into a retroviral virion. To produce these replication-defective retroviruses, retroviral packaging cell lines were developed (e.g., PA317, Ψ2, ΨAM) ***(2)***. These retroviral packaging cell lines contained an integrated retroviral genome, which lacked *cis*-acting packaging sequences (Ψ) necessary to package viral genomic RNA into a virion ***(1,2)***. Thus when a packaging cell line was transfected with a recombinant retroviral vector, it would release the recombinant retrovirus into the supernatant but the defective "helper virus" would not be packaged because it lacked necessary packaging (Ψ) sequences. Thus, these supernatants could be filter sterilized and used to efficiently infect cells. Development of amphitropic PA317 and ΨAM packaging cell lines allowed production of retroviruses which could infect a wide range of cells (human, mouse, cat, dog, and so on) because genomes of these packaging cell lines contain amphitropic *env* sequences ***(2)***.

The next breakthrough in this field occurred right after initial eukaryotic expression vectors containing dominant selectable markers were described ***(4)***. Now the concept of a retroviral vector containing a dominant selectable marker revolutionized gene transfer because it became possible to introduce genes into a large variety of cells with a diverse host range that had previously been difficult to transfect by calcium phosphate DNA precipitation, liposomes, or electroporation. Furthermore, antibiotic resistance genes enabled selection of retrovirally infected cells by culturing cells in the presence of an appropriate antibiotic. Cells could be selected for antibiotic resistance without selection for transformation or immortality, which were often traits selected for with nonrecombinant transforming retroviruses. High titers (10^6 focus forming units/mL) of recombinant retroviruses could be obtained by a ping-pong mechanism, which relies on passaging the viral supernatant between different retroviral packaging cell lines having different host ranges (PA317 → Ψ2 → PA317). Hence, reliable gene transfer for the scientific masses was available and one did not have to work on only fibroblastic cells.

Pitfalls of the retroviral vector technique included cDNA insert size, which usually had to be less than 5 kb. Furthermore, retroviral vectors based upon Moloney murine leukemia viruses (Mo-MuLV) required cells to be replicating in order for stable integration to occur. More recently, retroviral vectors based on the human immunodeficiency virus (HIV) genome have been designed which will infect and integrate (once the cell is activated) into the genome of quiescent cells ***(3)***.

An additional pitfall of retroviral vectors is that not every stably infected cell will express the gene of interest. Thus, it is possible that the gene one

wants to study will not be expressed despite antibiotic resistance gene expression. This may be caused by transcription control sequences, which regulate the particular gene. For example, cytomegalovirus (CMV) and simian virus 40 (SV40) promoter and enhancer elements may not readily support expression of particular genes in certain cell types. In contrast, the Mo-MuLV long terminal repeat (Mo-MuLV LTR) may be a stronger enhancer and promoter in many types of cells. Thus, one must confirm that the gene of interest is expressed by various assays. These assays include flow cytometric analysis, if the gene encodes a cell surface protein, analysis of transformation to factor independence, if the gene encodes a protein that relieves cytokine dependence, Western blot analysis if the gene encodes a protein distinguishable from endogenous proteins, or green fluorescence protein analysis (GFP), if the gene encodes a protein with a GFP tag.

Some of the first genes inserted into retroviral vectors were oncogenes, such as v-*src*, *BCR-ABL*, c-*myc*, and v-*Ha-Ras*, growth factors such as *IL-3* and *GM-CSF*, growth factor receptors such as *IGF-IR,* and anti-apoptotic genes such as *Bcl-2* ***(5–24)***. Genes inserted were often obtained from various plasmid vectors. With retroviral vectors, effects of wild type (WT) and mutant genes could be examined in other cell types. Studies performed with the *BCR-ABL* oncogene have indicated that it abrogates the cytokine-dependence of human and murine lymphoid and myeloid cells and that an autocrine component was often associated with transformation ***(7,13,18)***. In contrast, *BCR-ABL* does not induce transformation of NIH-3T3 cells ***(18)***. Other investigators have observed that recombinant retroviruses encoding v-*Ha-Ras*, v-*src*, *IL-3,* and other oncogenes abrogate cytokine-dependence of certain hematopoietic cell lines ***(6,7,13,19)***. However, a common pitfall of these studies is that abrogation of cytokine dependence may be indirect. The recombinant retroviral vector may insert adjacent to a gene to either enhance or diminish its function. If this proviral insertion event were required, it would indicate another genetic event was required for abrogation of cytokine dependence. A question commonly asked is whether multiple copies of the integrated gene are present in genomes of retrovirally infected cells. In most cases, infected clones contain only 1 to 2 proviruses (*see* **Fig. 5**). This is in contrast to cells transfected with plasmid DNAs which may, although not always, contain multiple copies of the introduced transgene ***(26,27)*** (**Fig. 4** in Chapter 16).

One of the more interesting genes inserted into a retroviral vector was a temperature sensitive (TS) v-*src* gene which functions at the permissive temperature of 35.5ºC, but not at the restrictive temperature of 39.5ºC ***(6)***. This retroviral vector encoding v-Src has allowed us to determine that transformation of certain murine hematopoietic cells was direct and dependent upon a functional v-Src oncogene ***(6)***. Furthermore we also ascertained that elevation of glucose transport in v-Src transformed cells depended upon functional v-Src ***(23)***.

The TS LA v-Src oncogene was isolated from a mutant v-Src retrovirus isolated in Los Angeles. cDNA encoding v-Src was inserted into a Mo-MuLV vector (pFGV) ***(6,23)***. This was one of the first uses of a Cond oncogene in a retroviral vector and it demonstrated that the v-Src oncogene could by itself abrogate cytokine dependence of this hematopoietic cell line because transformation was temperature sensitive. Similar studies were performed with TS v-Abl constructs ***(24)***.

In the early 1990s, the concept of a hormonally regulated oncogene became a viable approach to analyze signal transduction and malignant transformation ***(28–39)***. One of the first oncogenes inserted into a hormonally regulated retroviral vector was the c-*myc* gene ***(28)***. c-myc cDNA and the hormone binding (hb) domain of the human estrogen receptor (ER) were fused in frame to encode Myc:ER that could be regulated by β-estradiol. This chimeric protein allowed effects of c-Myc on cell growth and apoptosis to be analyzed. v-Src, v-Erb-B, and CR1, and CR2 domain deleted *Raf*-1 and *MEK*1 genes were similarly inserted into the ER constructs by Dr. Martin McMahon and his laboratory groups at DNAX and UCSF ***(29–32,37–39)***. Moreover, Dr. McMahon has developed plextrin homology (PH) domain deleted Akt constructs, which contain v-Src myristrylation and ER domains, which allows plasma membrane localization in addition to β-estradiol inducibility. A diagram illustrating key features of these and other retroviral constructs used to elucidate Raf/MEK/ERK and PI3K/Akt cascades is presented in **Fig. 1**. Many of these constructs are active in the presence of β-estradiol or the estrogen receptor antagonist 4-hydroxytamoxifen (4HT), but are inactive or much less active in their absence. The precise mechanism of activation of these constructs is unknown; however, a model for their activation is presented in **Fig. 2**. In this model, Cond constructs are inactive in the absence of 4HT with Hsp90 proteins bound to the hormone binding domains. Upon stimulation of cells with 4HT or β-estradiol, the Cond constructs dimerize and then perform their functions. Thus, activity of these constructs can be regulated with the appropriate hormone.

Recently, mutant ER binding domains have been created that bind 4HT 100-fold better than β-estradiol ***(39)***. These mutant ER constructs are preferred because one can stimulate cells with lower concentrations of 4HT than β-estradiol and potential actions of β-estradiol, such as proapoptotic or antiapoptotic effects, are avoided.

One pitfall of working with these constructs is that activation must be performed with phenol red free medium containing charcoal stripped FBS to remove steroids that may mimic β-estradiol. Phenol red free medium is used because phenol red has a conformation similar to estrogen and may activate ER constructs. Another pitfall associated with CR1 and CR2 deleted Raf and MEK constructs is that they lack some of the regulatory domains of these proteins, which

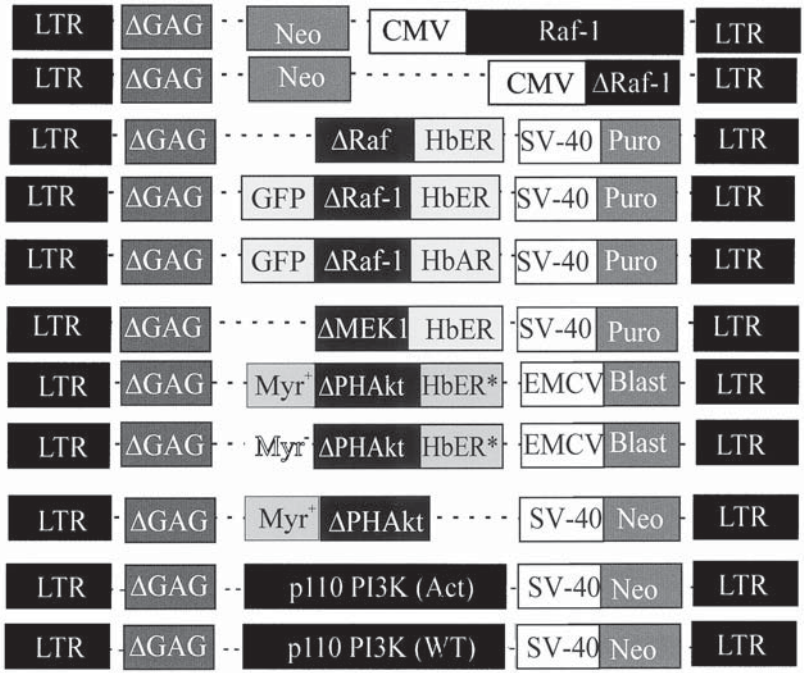

Fig. 1. Overview of Retroviral Vectors. *WT*, *CA*, and *Cond* forms of *Raf*, *PI3K*, and *Akt* genes have been inserted into different vectors encoding resistance to G418 (*neo*^{r}), puromycin (*puro*), and blasticiden (*blast*). The positions of the oncogene insert relative to the antibiotic resistances genes are indicated in this figure and whether the oncogene insert is derived from subgenomic mRNA. (CMV sequences control expression of Raf and ΔRaf-1). EMCV = internal ribosome entry sequence. Myr$^+$ = presence of v-Src encoded myristylation domain.

may shut off their activity. Certain interactions between Raf and regulatory proteins such as Ras and Akt may not occur in cells infected with ΔRaf:ER constructs because such regulatory sites are absent.

Other Cond Raf constructs have been made that encode CR1 and CR2 deleted Raf-1 fused to the androgen receptor (AR) hormone binding domain ***(36,40–42)***. One construct encodes a c-Myc tag and another encodes a GFP tag, which allows identification of these fusion proteins in infected cells ***(36,40–42)***. An advantage of the AR constructs is that one can use this construct to insert this hormonally regulated gene into breast cancer cells ***(40–42)***. Another advantage of these constructs is that one can introduce an ER construct (e.g., ΔAkt:ER) and then introduce an AR construct (ΔRaf-1:AR) into the same cells and then turn on one or both genes to follow their effects (by adding 4HT, testosterone

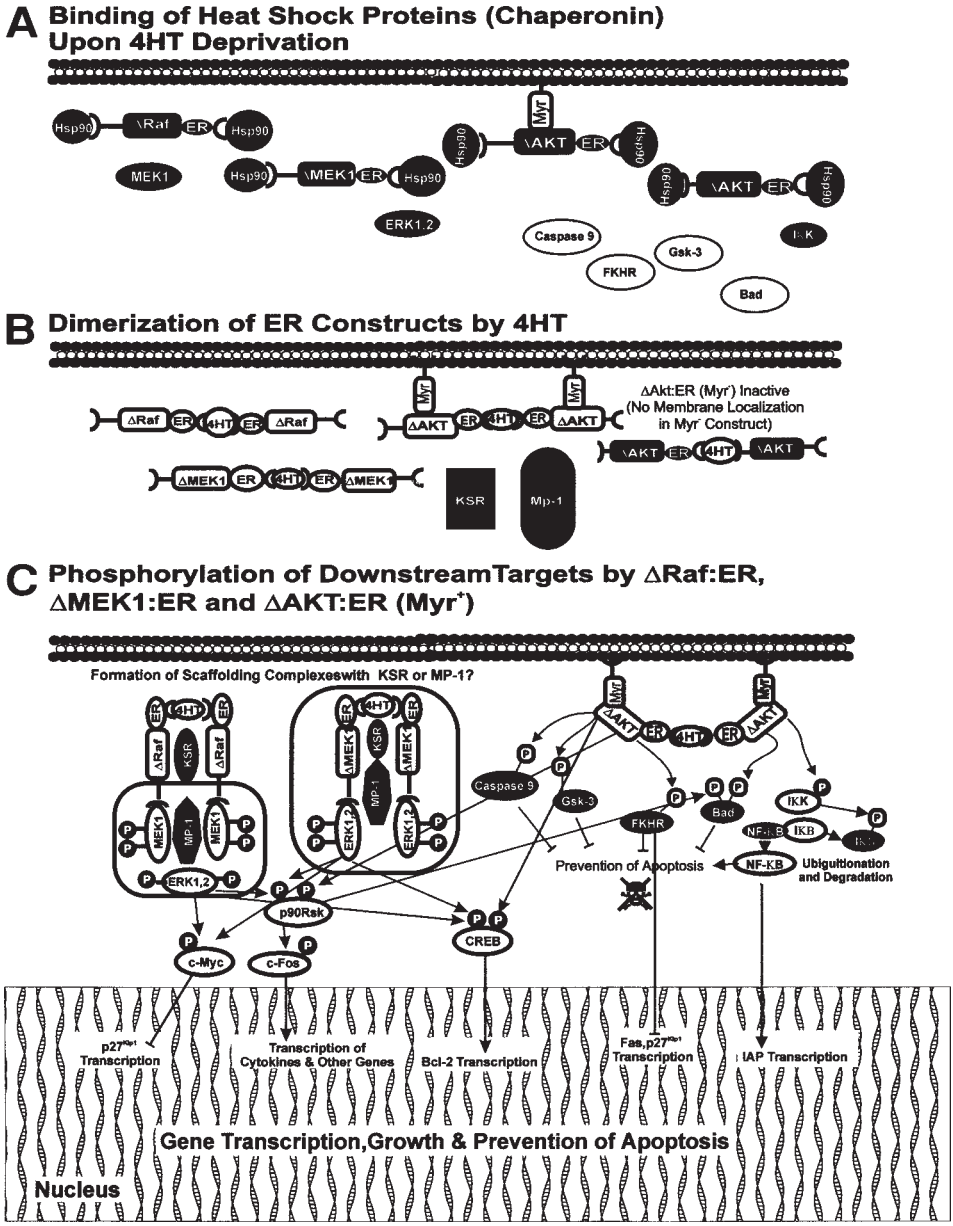

Fig. 2. Potential Mechanism of Activation of Oncogene ER Constructs. A potential mechanism of activation of ΔRaf:ER, ΔMEK1:ER, and ΔAkt:ER constructs by 4HT is indicated. In the absence of 4HT the constructs become inactivated. Potential target genes affected by activation of these Cond proteins such as $p27^{Kip1}$, Bcl-2, Fas, and IAP (inhibitor of apoptosis) are indicated.

or both). Recently, other hormonally regulating constructs have been made by using the progesterone receptor hormone binding domain ***(32,33)***.

Other modifications of signal transduction molecules have been made which render a protein kinase inactive (KI), kinase dead (KD), or dominant negative (DN). These different types of mutated genes as well as antisense (*AS*) genes have been introduced into retroviral vectors ***(14,43–49)***. Uses of *KI*, *KD*, *DN*, and *AS* genes include the ability to determine if their expression hinders oncogenes from transforming cells or to determine whether a particular pathway affects growth or apoptosis. Also, the KI and KD mutants can indicate whether the kinase activity of a protein is necessary for a given biochemical property. An immediate potential pitfall of using these types of inhibitory mutants is that they may have nonspecific effects due to their overexpression in cells and this caveat should be considered when using these vectors.

Many investigators only insert *KI*, *KD*, *DN*, and *AS* genes into plasmids which must be transfected into cells. While transfection occurs at adequate frequencies in fibroblastic cells, it is more difficult to transfect hematopoietic cells. The investigator should document that cells have indeed been transfected with these vectors and that they actually express the corresponding genes because a negative result is not readily interpretable. Some investigators have attached GFP or hemagglutinin (HA) tags to these constructs to enable identification of transfected or infected proteins within cells. Another approach is to use Cond constructs that encode proteins that can be controlled by an activating hormone. Expression of proteins encoded by Cond constructs can be demonstrated with an antibody to the hb domain. Dr. McMahon has used retroviral vectors encoding KI ΔRaf-1[301], which is a Cond mutant protein ***(14,32)***. This retroviral vector allows introduction of KI Raf-1 into many types of cells that are difficult to transfect, such as hematopoietic cells. Additionally, expression of KI ΔRaf-1 can be readily determined by Western blot analysis with an αER Ab.

Various investigators have attached the GFP moiety to their gene of interest ***(9,34,40)***. This can be advantageous for multiple reasons including; isolation of transformants by flow cytometric analysis, quantitatively demonstrating the amount of the protein expressed under different experimental conditions and finally, the localization of the transfected protein, i.e., does the protein localize to the cellular membrane, cytoplasm, nucleus or other membrane. Dr. McMahon has attached the GFP moiety to the conditionally active Raf protein ***(9,40)***. This has allowed us to quantitate GFP protein levels after transformation to cytokine-independence and after treatment with various signal transduction inhibitors to determine whether they affect Raf protein levels without having to perform labor intensive Western blotting experiments (*see* **Fig. 3**) ***(5,9,40)***. Other investigators have inserted the *GFP* gene in a construct encoding Bcl-2 ***(50)***. This construct allows isolation of cells that overexpress Bcl-2 by cell sorting with a

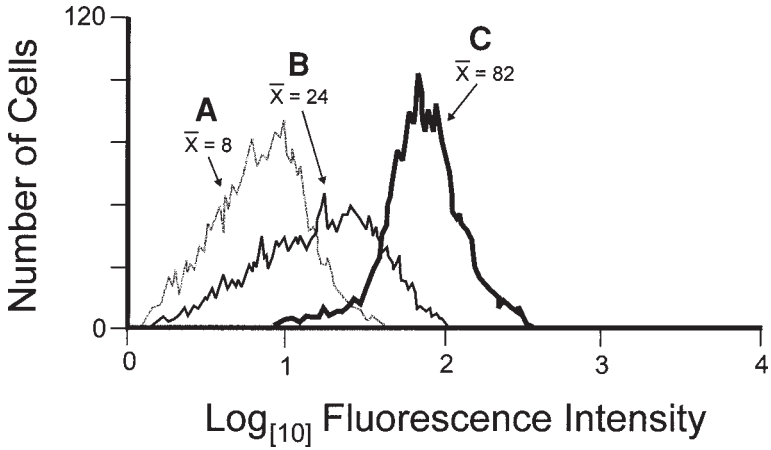

Fig. 3. Analysis of GFPΔRaf-1:ER Expression in Infected Cells. Analysis of GFPΔRaf-1:ER protein expression by flow cytometric analysis. The levels of the GFPΔRaf-1:ER were determined by flow cytometric analysis in (**A**) uninfected FL5.12 cells; (**B**) cytokine-dependent FL/GFPΔRaf-1:ER cells; and (**C**) 4HT-responsive FL/GFPΔRaf-1:ER + ΔAKT:ER cells.

FACS analyzer without the need for a dominant selectable marker gene. A potential pitfall is that some investigators have found that GFP may be toxic for certain cells and that over time GFP expression may decrease ***(36)***. In the stable transformants that we have isolated, we have observed that they maintain GFPΔRaf-1:ER expression in the absence of drug selection. We have also observed that the level of GFPΔRaf-1:ER expression was higher in the cells that grew in response to activated Raf, than those cells which remained cytokine-dependent, but even in the cytokine-dependent cells there was a low level of GFPΔRaf-1:ER expression which was not detected in the parental uninfected cells (*see* **Fig. 3**) ***(5,9)***. Moreover, we have observed that the GFPΔRaf-1:AR expression varies when cells are deprived of their growth stimulus, or treated with signal transduction inhibitors. These changes can be sensitively detected by flow cytometric analysis whereas such changes might be difficult to detect by western blotting or kinase assays ***(5,9)***. Thus addition of a fluorescent moiety to a protein may be a more sensitive, quicker and less expensive method to detect the level of the protein under different experimental conditions.

Over the past two decades our understanding of how cells transduce their growth and differentiation processes has evolved at an amazing rate, which has been due in part from the ability to modify genes and then transfer them into dif-

ferent types of cells. Knowledge of signal transduction pathways has shown us how complicated these processes are, yet has also provided us with novel targets for chemotherapeutic intervention.

2. Materials

2.1. Retroviral Vectors

Obtain the retroviral vector you desire to work with (*see* **Note 1**). There are many different retroviral vectors to choose from that encode different antibiotic resistance genes (e.g., *neo*^{r}, *puro*^{r}, *blast*^{r}, *hygro*^{r}). Under certain circumstances you may be able to obtain the retroviral vector containing the particular gene that you are interested in studying. In other cases, you may have to insert a WT or mutant gene into the multiple cloning site (MCS) of an empty retroviral vector. Some empty retroviral vectors, such as pMSCVneo^r, pMSCVhygro^r, and pMSCVpuro^r, and packaging cell lines, such as RetroPack® PT67, are commercially available from Clontech (Becton-Dickinson Clontech, Palo Alto, CA). To obtain others, contact the original investigator that developed them.

2.2. Cloning a Gene into a Retroviral Vector

1. DNA encoding the gene of interest contained in a plasmid or polymerase chain reaction (PCR) amplified.
2. Restriction endonucleases (REs) and RE maps of both the retroviral vector and the DNA of interest to design a strategy to introduce the DNA of interest into the retroviral vector in the correct orientation.
3. DNA ligase.
4. Competent bacteria such as DH5α (Invitrogen, San Diego, CA) for transformation with plasmid DNA.
5. Plasmid purification kit (Qiagen, Valencia, CA).
6. Agarose (Sigma, St. Louis, MO).
7. Horizontal electrophoresis apparatus (e.g., 10 × 7 cm) (Invitrogen or BioRad, Hercules, CA).
8. 10 mg/mL Ethidium bromide stock solution wrapped in foil because it is light sensitive (ethidium bromide has a working solution concentration of 500 ng/mL and is carcinogenic).
9. Ultraviolet (UV) transilluminator with gel documentation system to determine whether the DNA of interest has been inserted into the retroviral vector in the correct orientation.

2.3. Retroviral Packaging Cell Lines

1. Retroviral packaging cell line such as PA317 [American Type Tissue Collection (ATTC), Rockville, MD], ψ2, ψAM, gp+E86 (ATTC), BOSC23, Phoenix, 293T, or RetroPack® PT67 (BD Biosciences Clontech).

2. Cell culture media for retrovirally infected cells such as minimal essential Eagle's medium (MEM), Dulbecco's modified Eagle's medium (DMEM), or Iscove's modified Dulbecco's medium (IMDM) with heat inactivated 5% to 10% FBS or BCS (most media can be purchased as 10X powder mixtures from Invitrogen and one can make 1X media with purified water).
3. 1X Trypsin solution (Invitrogen).
4. 15-mL sterile centrifuge tubes to centrifuge cells after trypsin treatment.
5. 60-mm tissue culture plates, as well as T25 and T75 tissue culture flasks (Corning, Corning, NY) to plate out cells in.

2.4. Transfection of Retroviral Packaging Cell Lines

1. Lipofectin® or Lipofectamine® (Invitrogen).
2. Sterile glass disposable pipets.
3. Opti-MEM-I medium (Invitrogen).
4. Antibiotics: G418 (Invitrogen), blasticiden (Invitrogen), puromycin (Sigma), hygromycin (Calbiochem, San Diego, CA).
5. 1X Trypsin solution (Invitrogen).
6. 15-mL sterile centrifuge tubes to centrifuge cells after trypsin treatment.
7. 60-mm tissue culture plates and T25 and T75 tissue culture flasks to plate out cells in.
8. Pasteur pipets to aspirate media from Petri dishes.
9. Vacuum source: either in house vacuum or vacuum pump connected to an Erlenmeyer aspiration flask.

2.5. Infection of Adherent and Nonadherent Cells

1. Cells of interest.
2. Media to grow cells of interest.
3. Growth factors or cytokines for cells of interest, if necessary.
4. Sterile 60-mm tissue culture plates or 96-well flat bottom culture plates (Corning).
5. Adjustable pipetter (Eppendorf, Eppendorf, Germany) and pipet tips.
6. Antibiotics and tissue culture flasks as described above in **Subheading 2.4.**
7. Filter sterilized 1 mg/mL Polybrene® (hexadimethrine bromide) (Sigma) dissolved in water.
8. Acrodisc 0.45-μm syringe filters (PALL Gelman Laboratory, Ann Arbor, MI) to filter sterilize cell supernatants containing retrovirus.
9. 150-mL bottle top filter sterilizing units (0.45 μ*M*, Corning).

2.6. Equipment for Analysis of Construct Inheritance and Expression

1. PCR machine.
2. Liquid scintillation counter.
3. Gel electrophoresis equipment for Southern, Northern, and Western blot analysis.
4. Flow cytometer.

3. Methods

3.1. Cloning a Gene Into a Retroviral Vector

Digest the plasmid DNA containing the cDNA encoding the gene you are interested in working with, with the appropriate RE to excise the complete cDNA sequence. Purify the cDNA insert by electrophoresis followed by excising the full-length sequence from the DNA agarose gel. Insert the cDNA insert into the retroviral vector plasmid DNA and ligate the cDNA insert in the retroviral vector with DNA ligase. Transform appropriate bacterial strain (DH5α, Invitrogen). Harvest plasmid DNA from bacteria. Confirm full-length cDNA has been inserted into the retroviral sequence in the correct orientation.

3.2. Transfection of Retroviral Packaging Cells with Retroviral Plasmid DNA

1. Seed appropriate adherent cell line in 60-mm plates at 2×10^5 to 5×10^5 cells/plate (approx 50% to 75% confluent) one day in advance.
2. Determine the DNA concentration, then aliquot 25 to 50 µg DNA in sterile 12 × 75 mm polystyrene tubes. The optimal amount of DNA should be titrated for each cell line because some cell lines are sensitive to the relatively large amounts of DNA indicated above, however, if insufficient DNA is used you will not get optimal levels of liposome formation and DNA transfer.
3. Adjust volume of DNA to 100 µL with sterile double distilled purified water.
4. Include as one of your samples a negative mock control (e.g., no plasmid DNA containing antibiotic resistance marker) to ensure that the antibiotic concentration will kill off all the non-transfected cells.
5. Add 100 µL of Lipofectin® (equal volume as DNA solution), with sterile glass disposable 1-mL pipet, to the tube containing 100 µL DNA, mix gently by pipeting slowly. You should see a cloudy solution. This is the DNA:liposome complex. If you do not see this cloudy solution, it may indicate your DNA concentration is too low and you need to increase it.
6. Incubate the DNA:liposome mixture for 15 to 30 min at room temperature in the sterile tissue culture hood. In the meantime, warm at 37ºC a bottle of Opti-MEM-I medium (Invitrogen).
7. At about the end of the 15 to 30 min incubation, remove the medium from the fibroblasts cultured in the Petri dish by aspiration with a sterile Pasteur pipet. Wash the cells twice with 2 mL of Opti-MEM medium. Finally, remove the Opti-MEM-I medium from the plate. Opti-MEM-I has low serum and lacks antibiotics.
8. Gently add 1 mL of Opti-MEM-I medium to the DNA:Lipofectin® mixture by dribbling the Opti-MEM-I down the tube. Gently resuspend by drawing the mixture up the pipet.
9. Add dropwise the Opti-MEM-I:DNA:liposome mixture to the Petri dish containing cells that has had the tissue culture medium removed.

Table 1
Drug Concentrations for Selection of Stably Infected Cells

	Type of Cells		
Antibiotic	Murine Fibroblast	Murine Hematopoietic	Human Hematopoietic
G418	500 µg/mL to 1000 µg/mL	2 mg/mL	2 mg/mL
Puromycin	4 µg/mL	1 to 2 µg/mL	1 to 2 µg/mL
Blasticiden	25 µg/mL	25 to 50 µg/mL	25 to 50 µg/mL
Hygromycin	200 µg/mL	400 µg/mL	400 µg/mL

10. Gently rock the plate to spread the Opti-MEM-I:DNA:liposome mixture over the plate, put in CO_2 incubator for 4 to 6 h. At the end of the incubation add 3 mL of normal medium containing serum to the plate. Incubate for 2 d.
11. Add normal medium containing serum and selective antibiotic (*see* **Table 1** for concentrations), change medium every 3 d after aspiration of the old medium from the plate.
12. After 8 to 12 d of incubation, foci of transfected cells should be visible on the bottom of the plate. Pools of transfected cells can be isolated from these foci. Pools of 100 to 200 foci with approx 100 to 1000 cells/foci are preferred over clones because they usually yield a higher titer of recombinant retrovirus. Aspirate medium from tissue culture flask, wash cells with 1X PBS to remove residual medium, treat cells with 1X trypsin for 5 min, recover cells by pipetting, then transfer cells into a 15-mL centrifuge tube.
13. Centrifuge cells at 1000*g* for 5 to 10 min, then discard the supernatant. Be careful because cells may be present as a cloudy haze in the tube instead of a pellet. If no cell pellet is obtained, centrifuge again until a cell pellet is formed. Resuspend transfected cells in growth medium then plate into a T75 flask with 10 mL of medium (*see* **Note 2**). When the cells become 50% to 75% confluent, add fresh medium and harvest the medium daily. This is the primary viral supernatant (first passage) (*see* **Note 3**).
14. The viral supernatant may be stored at −80ºC or used immediately to infect cells. Filter sterilize the viral supernatant with an Acrodisc 0.45-µm syringe filter prior to infection of cells.

3.3. Infection of Retroviral Packing Cells with a Recombinant Retrovirus

1. Infect fibroblastic cells in 60-mm Petri dishes at 2×10^5 to 5×10^5 cells/dish with 1 mL of filter sterilized viral supernatant and 1 µL of 10 mg/mL Polybrene® (final 10 µg/mL) (1000X stock, store aliquots at −20ºC). Polybrene® enhances viral

penetration into cells. Cells should be seeded the day previous at 50% to 75% confluence with fresh medium. Mo-MuLV based retroviruses infect and integrate into cells best in S phase.

2. Incubate for 4–6 h, add regular medium back to cells, then incubate for an additional 48 h to allow integration of the retroviral provirus into cellular DNA. Select cells with an appropriate antibiotic that corresponds to the resistance marker contained in the retroviral vector, then make pools or clones as described in previous procedures. Antibiotic concentrations for selection of transfected cells are presented in **Table 1**.
3. Allow cells to grow for 8 to 21 d, trypsinize and pool foci together, then culture cells in a T75 tissue culture flask in the presence of an appropriate selective antibiotic. Allow cells to reach 50% to 75% confluence. Viral titers decrease substantially when fibroblasts are confluent because murine retroviruses are produced at highest titers when cells are cycling. Add fresh medium lacking selective antibiotic. Collect the supernatant, which contains the retrovirus 1 d later. Murine based retroviruses will be produced at highest titers 24 h after the cells reach 50% to 75% confluence. You can add back fresh medium to the flask so that you can collect more viral supernatant the next day.
4. Either store the non sterile viral supernatant in a −80ºC freezer or filter sterilize the viral supernatant 0.45-µm syringe filter. Supernatants from different days from the same infected line can be pooled together and filter sterilized with a bottle top filter (0.22 µ*M* bottle top filter, Corning) and then aliquoted into 15-mL sterile centrifuge tubes and stored in the −80ºC freezer until you are ready to use them. Do not do repeated freeze/thaw cycles on the retroviral stocks as they decrease by a factor of approximately ten everytime you freeze and thaw the stock.
5. Pass the virus through another retroviral packaging cell line, which contains a different *env* sequence. This is done to increase the retroviral titer (e.g., first transfect amphitropic PA317 cells with plasmid DNA, second infect ecotropic Ψ2 cells with viral supernatant from transfected amphitropic PA317 cells, third infect amphitropic PA317 cells with supernatant from Ψ2 cells). Retroviral titers are initially very low. This is in contrast to other viruses such as adenoviruses. This ping-pong mechanism amplifies the retroviral titer. Passage through a cell line with a different *env* sequence is believed to increase the titer of the retrovirus.
6. Titer the retrovirus on a fibroblastic cell line that does not contain the dominant selectable marker gene, which the retrovirus does contain. Add serial tenfold dilutions of retroviral stock to fibroblasts cells. Two days post-infection, add medium containing the respective antibiotic (e.g., G418, puromycin, hygromycin, or blasticiden). Change the medium every 3 d. After about 2 wk you should see colonies (foci) of cells growing on the bottom of the plates, confirm under the microscope. Stain the plate with a dye that will stain the cells such as Giemsa stain (Gurr BDH Chemicals, LTD., Poole, England) for 10 to 30 min, gently wash off the dye in the sink with water. Count number of foci per plate. Usually, the dilution yielding 5 to 25 colonies is the most informative for determining the titer of a retrovirus.

3.4. Commercial Liposomes to Transiently Introduce DNA into Retroviral Packaging Cells

1. Purify plasmid containing the retroviral vector from bacteria.
2. Plate 5×10^6 retroviral packaging cells (e.g., PA317 or BOSC23) into 100-mm dishes 1 d prior to transfection.
3. Mix 10 µg DNA with 1 mL of Opti-MEM-I medium.
4. Mix 90 µL Lipofectamine® (Invitrogen, San Diego, CA, USA) with 1 mL Opti-MEM-I medium.
5. Mix the two solutions gently by pipeting them together in a polystyrene tube.
6. Incubate the mixture for 15 to 30 min.
7. Wash retroviral packaging cells twice, each with 4 mL Opti-MEM-I medium.
8. Add the DNA/Lipofectamine® mixture to cells, incubate for 4 to 6 h, remove the medium, then wash the plate once with Opti-MEM-I medium because it may be toxic to certain cells.
9. Add 8 mL of medium (DMEM + 10% FCS lacking antibiotics), then incubate for 18 to 20 h.
10. Remove medium, then add 5 mL of fresh medium (DMEM + 10% FCS lacking antibiotics).
11. Incubate for 24 h, then filter sterilize the medium, which is the viral supernatant. Either use viral supernatant for infection or store in a −80ºC freezer until use. Avoid repeated freeze thaws of supernatant, because that will decrease viral titer. Transient transfections yield high titers of recombinant retroviruses.

3.5. Stable Infection of Nonadherent (Cytokine Dependent) Hematopoietic Cells (Outlined in Fig. 4)

1. Retroviral infection of nonadherent cells is slightly different from retroviral infection of adherent cells. Collect the cells by centrifugation at 500*g* for 5 min, dispense the cells into 12 × 75 mm tubes (Fisher Scientific, Pittsburgh, PA) with retroviral stocks and Polybrene® (*see* **Subheading 3.5.2.**), then plate cells into either plates or flasks. Cells should be fed the day before infection with fresh medium and cell density should not be too high. Cell density should be approx 2×10^5 cells/mL to ensure cells are cycling because they are most sensitive to retroviral infection when in the S cell cycle phase.
2. Cells are collected and 2 mL containing 2×10^5 cells is mixed with 1 mL of viral supernatant and 3 µL of 1000X Polybrene® stock solution. The mixture is resuspended, cells are plated in 100-mm Petri dishes, then the plate is gently rocked to spread out the cells. Cells are incubated in the CO_2 incubator for 4 to 6 h. Different viral supernatant dilutions may be used.
3. 7 mL of medium is added, then proviral integration is allowed to proceed by incubating for an additional 48 h (outlined in **Fig. 4, Panel 2, B**). Cells are transferred to 15-mL tubes, then centrifuged at 500g for 5 min.
4. Cells are resuspended in 10 mL of medium containing the appropriate antibiotic (*see* **Table 1**), then 100 ml is added to each well of a 96-well plate with a multichannel

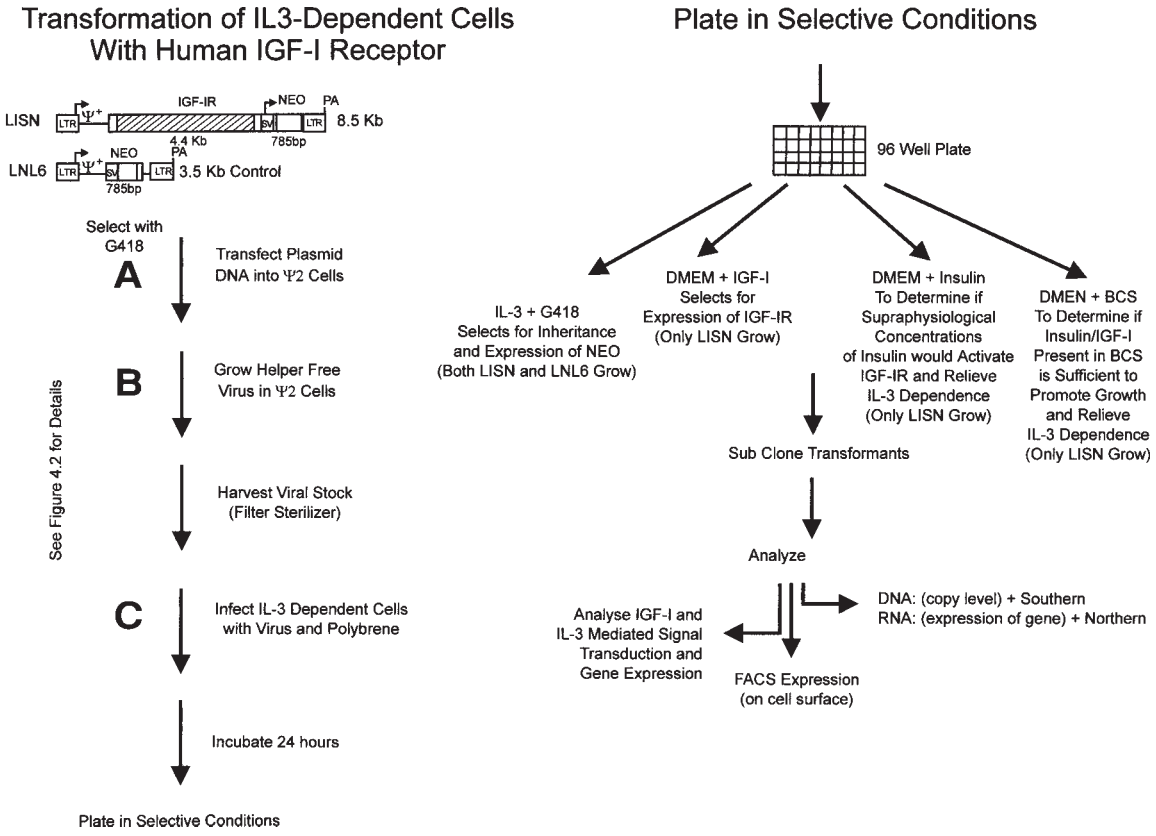

Fig. 4. Infection and Selection of Hematopoietic Cells with hIGF-IR Retrovirus. (Panel **4.1**) An overview of methods to infect hematopoietic cells with a retrovirus containing a potential oncogene and to select for cells transformed to grow in response to that introduced oncogene is presented.

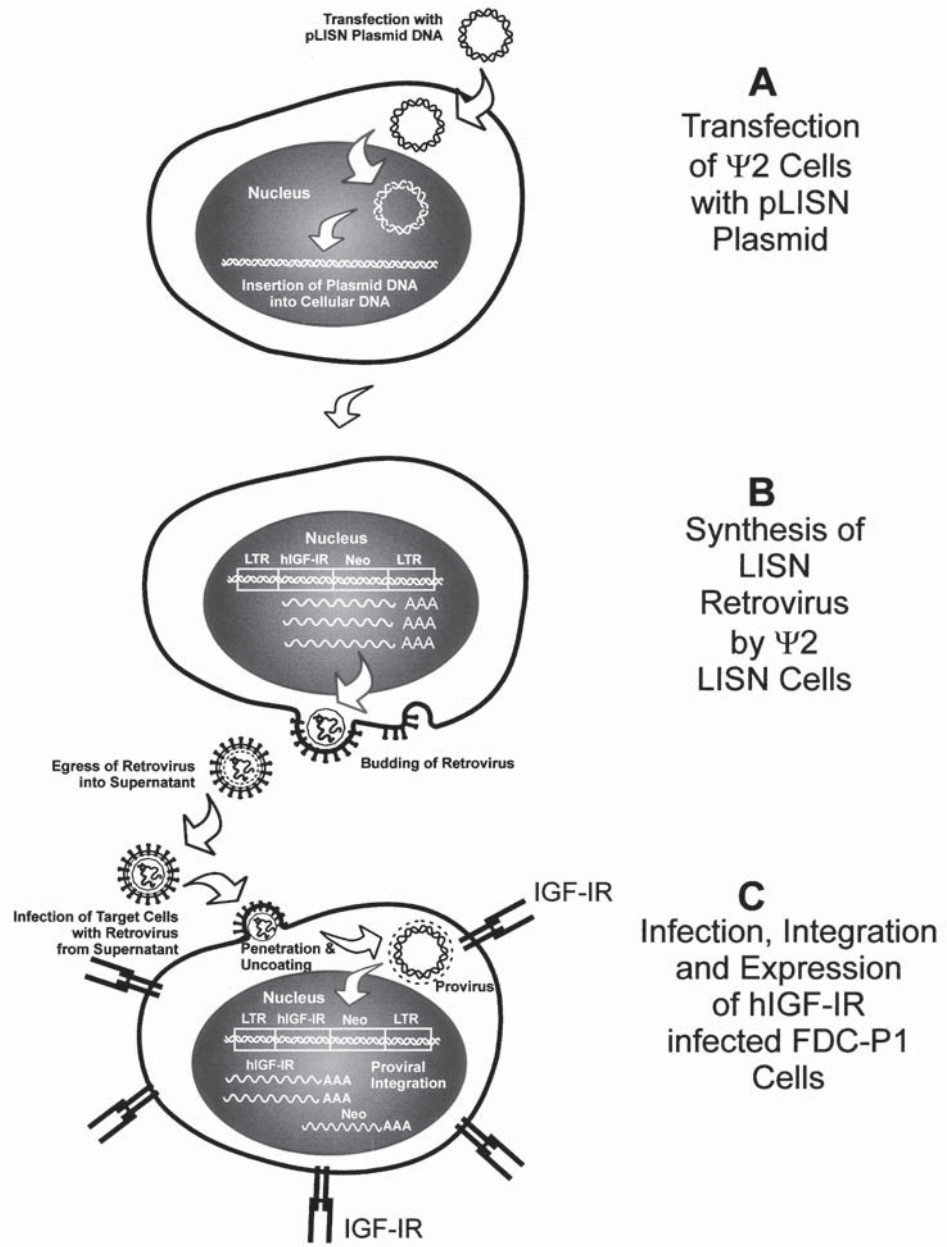

Fig. 4. (Panel **4.2**) An enlargement of steps **A–C** is present to illustrate transfection of the packaging cell line, recombinant retroviral synthesis and subsequent infection of a target cell line.

pipet (at a 100-µL setting). Alternatively, if different selection conditions are used, cells may be split into different tubes in advance of various treatments (*see* **Note 4**).

5. Cells are fed 100 µL of fresh medium every 3 d. After the second feeding on the sixth day of incubation, some medium can be removed by withdrawing 150 µL with a multichannel pipeter without touching the bottom of the dish. Add 100 µL of fresh medium containing selective antibiotics. Depending on how many wells of the 96-well plate are positive for growth, either individual clones or pools of infected cells will be obtained. If clones are desired, subcloning experiments may be necessary.
6. It is essential to also perform a mock infection to insure that the drug concentration is sufficient to kill off all uninfected cells. If working with cytokine dependent cells, one may have to include the cytokine in the medium. If interested in abrogating cytokine dependence, one can remove cytokine from the medium when cells are seeded in 96-well plates. If working with a *Cond* oncogene, one can include the compound that induces the oncogene (β-estradiol, 4HT, or testosterone) in the absence of cytokine, but in the presence of a selective antibiotic.
7. Characterize cells to ensure they have inherited and express both the drug resistance gene and the gene of interest that are contained in the retroviral vector. One can test for inheritance of the retrovirus by performing Southern blot analysis on DNA isolated from infected cells (*see* **Note 5**). Alternatively PCR can be performed.
8. Do not assume that infected cells will express the gene you have inserted into the vector unless it confers a growth advantage to the cells (*see* **Notes 6–10**).
9. Determine the effects that introduction of your gene has on various signal transduction, apoptotic, and drug resistance pathways operating within cells. This may be the ultimate goal for performing these experiments (*see* **Notes 11–20**).

4. Notes

1. Plan ahead when deciding which retroviral vector to work with because one may want to subsequently retrovirally infect cells with a second gene. The retroviral vector containing the second gene will require a different selectable marker. If infecting cells with one retroviral vector, a vector encoding G418^r (*neo*^{r}) is preferrable because G418 is the easiest antibiotic to work with and most infected eukaryotic cells grow well in it. Other antibiotics (puromycin, hygromycin, blasticiden, and mycophenolic acid) are more difficult to work with because there is often a fine line between killing all cells versus killing only uninfected cells.
2. To ensure that you have isolated transfected cells, you may include the selective antibiotic (G418, puromycin, hygromycin, blasticiden) in the pooled culture which you have expanded after resuspending the pellet recovered after trypsin treatment treatment. However, 1 d prior to the recovery of viral supernatants, you should remove the antibiotic from the culture conditions.
3. The titer of the virus recovered after the initial transfection is usually low and should be amplified by passage on another retroviral packaging cell line with a different retroviral *env* sequence.

4. We next present some examples of how retroviral vectors containing oncogenes can be used to investigate cellular transformation. An example of the effects of a *WT* gene on the transformation of cells is shown by studies that we performed to determine the ability of overexpression of the IGF-IR to abrogate the cytokine–dependence of hematopoietic cells. We have described this example to illustrate some of the experimental techniques, which one may want to perform to elucidate the effects of a gene on the transformation of cells. The IGF-I/IGF-IR interaction was previously shown to be an important system involved in the regulation of organismal growth, therefore, we wanted to determine whether it could also be an important ligand-receptor system involved in malignant transformation of hematopoietic cells. An outline of this experiment is presented in **Fig. 4**. The following experiments were performed to test the hypothesis that overexpression and activation of the IGF-IR would abrogate the cytokine-dependence of certain hematopoietic cells by a ligand-dependent mechanism.

   First, two retroviral vectors were obtained from Dr. Michael Kaleko and A. Dusty Miller from the Fred Hutchinson Cancer Research Center in Seattle, WA. LISN encodes the full length IGF-IR and LNL6 is an empty retroviral vector ***(52)***. IL-3 dependent FDC-P1 cells were infected with the retroviral stocks as described previously in **Subheading 3.5.** 24 to 48 h later, the cells were plated in the indicated selection conditions (*see* **Fig. 4**). All selection conditions included DMEM + 5% BCS and A) G418 + IL-3, in which both the LISN and LNL6 infected cells should grow; B) G418 + IGF-I in which the LISN-infected cells might grow in response to activation of the IGF-IR; C) G418 and supraphysiological concentrations of insulin in which the LISN-infected cells might grow in response to activation of the IGF-IR (insulin at high concentrations will bind and activate the IGF-IR) and, finally; D) G418 in which the endogenous insulin or IGF-I present in the BCS might activate the IGF-IR at sufficient levels for growth.

   LISN and LNL6 infected cells were readily recovered in medium containing G418 + IL-3, indicating that overexpression of the *neo*^{r} gene present in the two vectors allowed the cells to grow in medium containing G418 ***(20)***. However, mock-infected cells did not grow in these culture conditions indicating that the G418 concentrations were sufficient to kill the uninfected parental cells. In contrast only LISN (and not LNL6) infected cells grew in DMEM containing IGF-I, insulin, or just BCS indicating that overexpression of the IGF-IR allowed the cells to grow in these selection conditions. Fewer cells were recovered from cells selected in the presence of just DMEM + BCS indicating that the endogenous insulin/IGF-I present in BCS was not optimal for inducing growth in response to IGF-IR overexpression ***(20)***.
5. Inheritance of LISN and LNL6 proviruses was demonstrated by Southern blot analysis of DNA isolated from uninfected FDC-P1 cells as well as FDC-P1 cell clones infected with LISN or LNL6 retroviruses (*see* **Fig. 5**). Kpn-1 cleaves within the MCS of LISN to release a 4.4 kb hIGF-IR fragment. This internal 4.4 kb hIGF-IR fragment is present in FDC-P1 cells infected with the LISN retrovirus, but not in

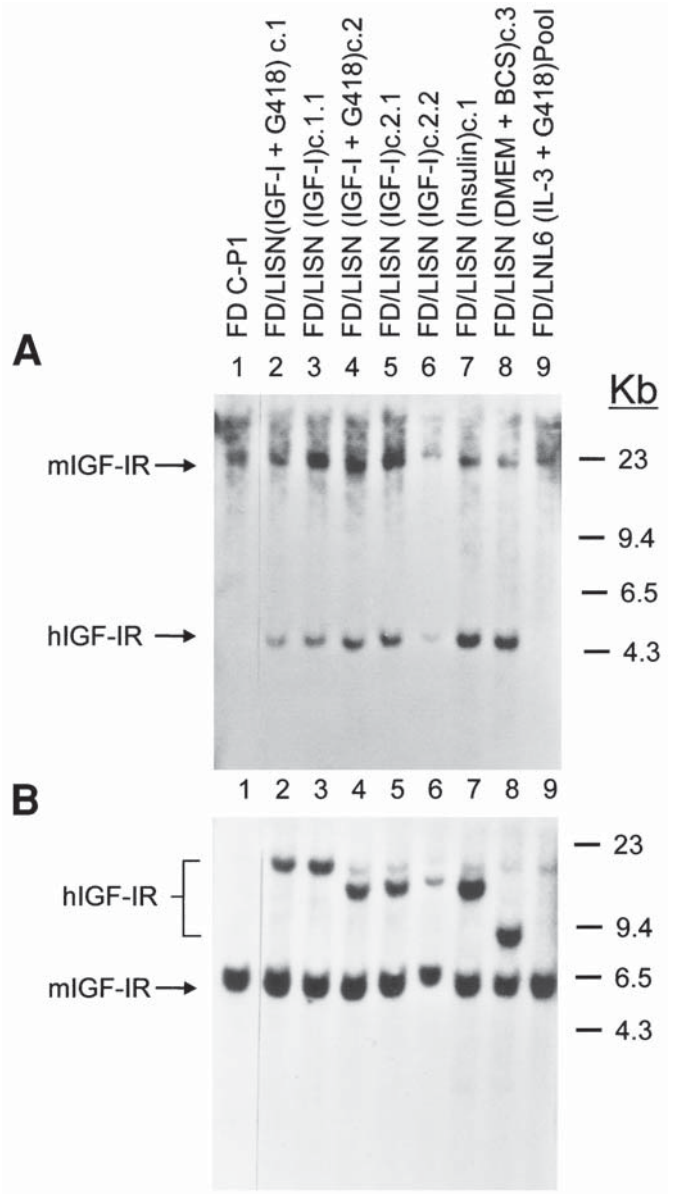

Fig. 5. Inheritance of *hIGF-IR* Provirus in Infected Cells. Southern blot analysis on Panel (**A**) Kpn-I and Panel (**B**) EcoRI digested DNA. DNA filters were hybridized to an IGF-IR cDNA insert.

uninfected FDC-P1 cells or FDC-P1 cells infected the LNL6 retrovirus. Clones need not be isolated because an enzyme like Kpn-1 allows one to confirm inheritance of LISN provirus in pools of infected cells by releasing an internal fragment. The intensity of the internal fragment can be compared to the intensity of a cross hybridizing fragment (e.g., mIGF-IR) to estimate the number of proviruses inherited by the cells. In this case, cells inherited 1–2 copies of hIGF-IR provirus.

To determine whether the clones are unique, one can use a RE, which cleaves once in the retroviral construct such as *Eco RI*. Cleavage with this enzyme should yield different sized fragments depending upon where the provirus integrated as one EcoRI fragment is present in the integrated provirus and the next *Eco RI* site is present in the adjacent cellular DNA. As seen in the bottom panel of **Fig. 9**, the LISN provirus sites of integration are unique in the subclones that were derived from one another, but different in the clones, which were not derived from one another.

6. The expression of the retroviral construct can be determined by Northern blot analysis as shown in **Fig. 6**. The top panel of **Fig. 6** demonstrates expression of full-length hIGF-IR mRNA in was detected with a *hIGF-IR* cDNA probe in LISN-infected cells, but not in LNL6-infected or uninfected FDC-P1 cells. The middle panel of **Fig. 6** shows that a *neo*^{r} cDNA probe detected full length and subgenomic *neo*^{r} transcripts in LISN infected cells with sizes of 8.5 and 0.8 kb, respectively. Full length 3.5 kb *neo*^{r} mRNA was detected in LNL6-infected cells. In contrast, no *neo*^{r} transcripts were detected in uninfected FDC-P1 cells. Thus, LISN infected cells expressed integrated LISN provirus.
7. To determine whether hIGF-IR was detected on the cell surface of LISN infected cells, FACS analysis was performed with an antibody (IR3) that recognizes hIGF-IR (*see* **Fig. 7**). hIGF-IR was readily detected by FACS analysis, while only background levels were detected in FDC-P1 cells infected with an empty retroviral vector. Higher levels of hIGF-IR were detected on the cell surface of cells that grew in response to IGF-I/insulin present in the BCS.
8. The number of IGF-IR receptors on the cell surface can be determined by radiolabeled ligand analysis followed by Scatchard analysis (*see* **Fig. 8**). Higher numbers of IGF-IR were detected on the LISN-infected cells selected in DMEM + BCS than in cells selected in IGF-I (Panel A) or insulin (Panel B), as well as the parental FDC-P1 cells or cells infected with LNL6 (Panel C).
9. The effects on the overexpression of the IGF-IR on the growth of the cells were determined. Overexpression of IGF-IR promoted growth in a ligand–dependent, dose-dependent fashion in the LISN infected cells selected in IGF-I but not in the uninfected cells (*see* **Fig. 9**). Interesting the LISN-infected cells selected for growth in BCS alone grew in response to BCS but importantly their growth was also inhibited by the αIGF-IR Ab.
10. The ability of the LISN infected cells to form tumors in immunocompromised mice was determined. The FD/LISN cells formed tumors in 16/16 mice (four clones tested) whereas control cytokine-dependent cells did not (0/12, three clones tested). Thus these cells represent a good model to understand IGF-I/IGF-IR mediated signaling, prevention of apoptosis and induction of malignant transformation.

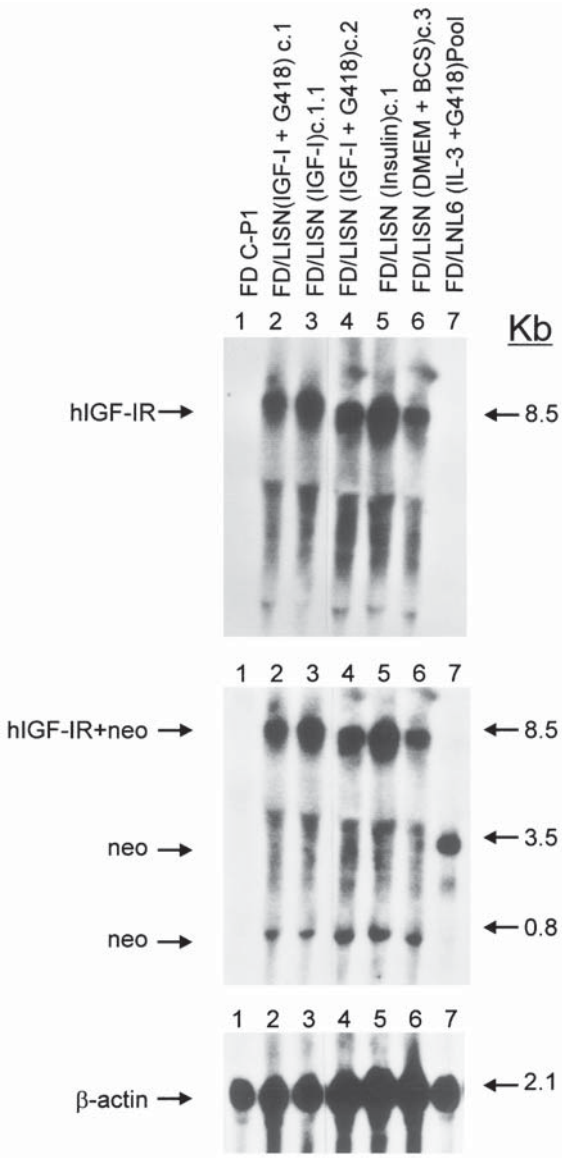

Fig. 6. Expression of *hIGF-IR* and *neo*^{r} mRNA Transcripts in Infected Cells. Northern blot analysis was performed to determine whether the cells expressed IGF-IR and *neo*^{r} transcripts. The levels of β-actin were determined in the cells as a RNA loading control.

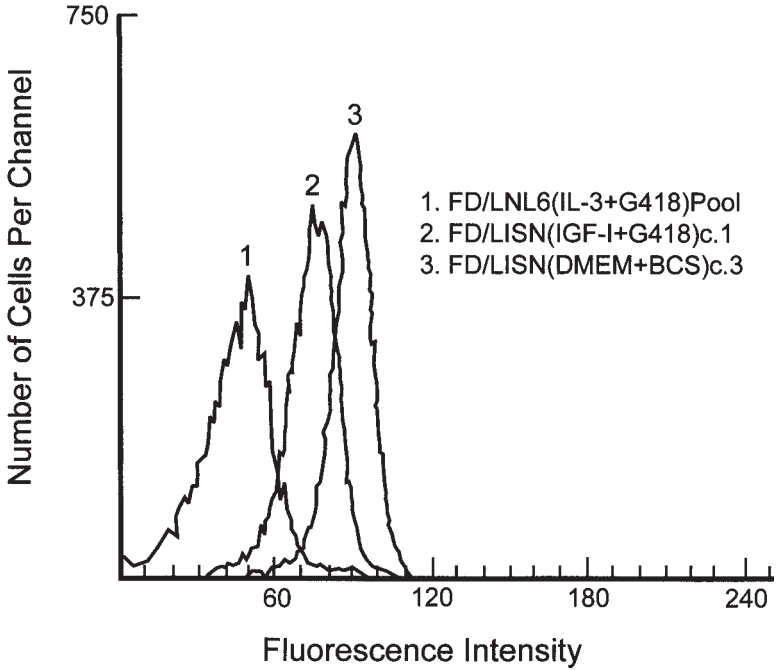

Fig. 7. Expression of *hIGF-IR* on the Cell Surface in Infected Cells. FACS analysis of IGF-IR expression was determined by staining the cells with an αIR-3 MoAb which specifically recognizes the human IGF-IR. Higher levels of hIGF-IR expression were observed in the FD/LISN (DMEM + BCS)c3 cells which grew in response to IGF-I/ insulin contained in the FBS.

11. An example of how *CA* and *Cond* oncogenes can be used to determine the effects of an oncogene on transformation of hematopoietic cells is our studies performed with the *Raf* oncogene and cytokine-dependent hematopoietic cells. The *WT Raf*-1 containing retrovirus does not induce malignant transformation of NIH-3T3 cells or abrogate the cytokine dependence of hematopoietic cells ***(8,14)***. However, a *CA Raf*-1 construct (lacking negative regulatory CR-1 and CR-2 domains) will transform NIH-3T3 cells and abrogate the cytokine dependence of murine FDC-P1 cells and human TF-1 cells ***(8,14)***. The hematopoietic cell lines produce autocrine GM-CSF, presumably due to Raf activating the downstream ERK1 and ERK2 proteins, which phosphorylate $p90^{Rsk}$, which in turn phosphorylates certain transcription factors including CREB and ELK, which bind regulatory sequences in the GM-CSF gene and induce its transcription. Moreover, ΔRaf-infected NIH-3T3 and MCF-7 cells also produce autocrine growth factors ***(39,41)***.
12. An advantage of the CA constructs is that one can determine if the oncogene transforms the cells to a state where they induce the malignant transformation of the cells because it is possible to inject the cells infected with the *CA* and *WT Raf* genes into immunocompromised mice and determine whether the cells form tumors ***(8)***.

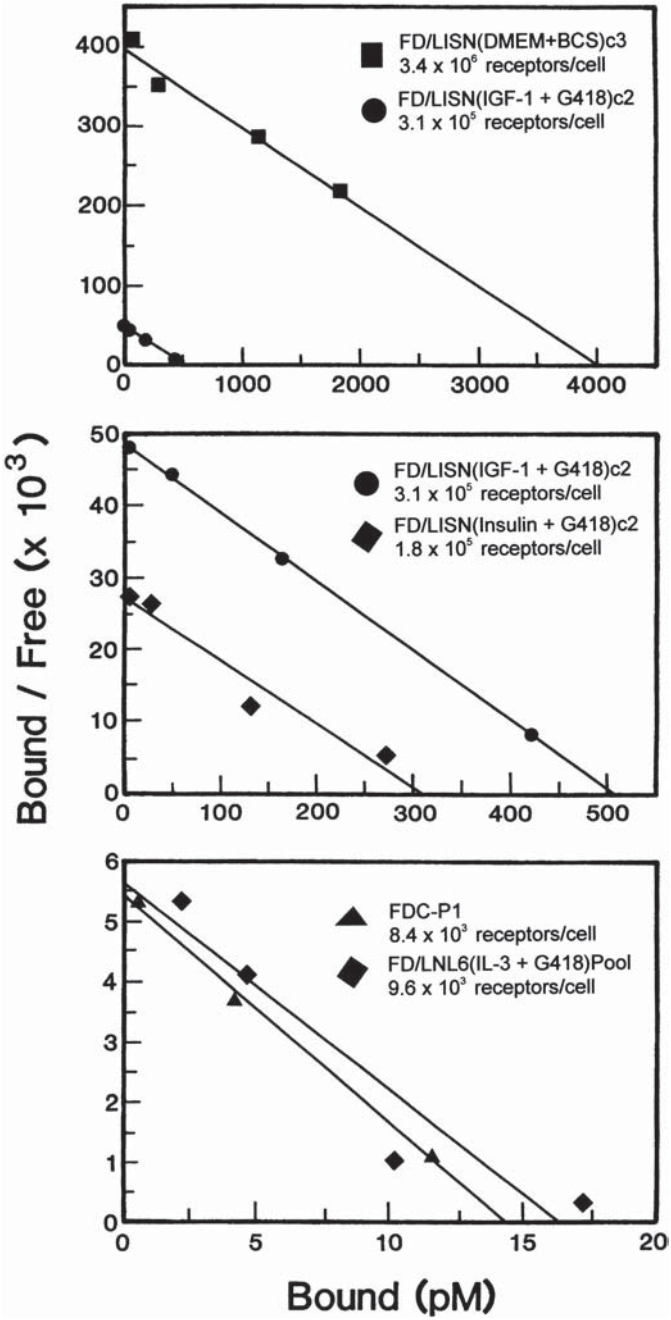

Fig. 8. Estimation of hIGF-IR Expression by Ligand Binding Analysis. The number of receptors was estimated by radiolabeled IGF-I binding experiments. Scatchard analysis was used to estimate the number of receptors.

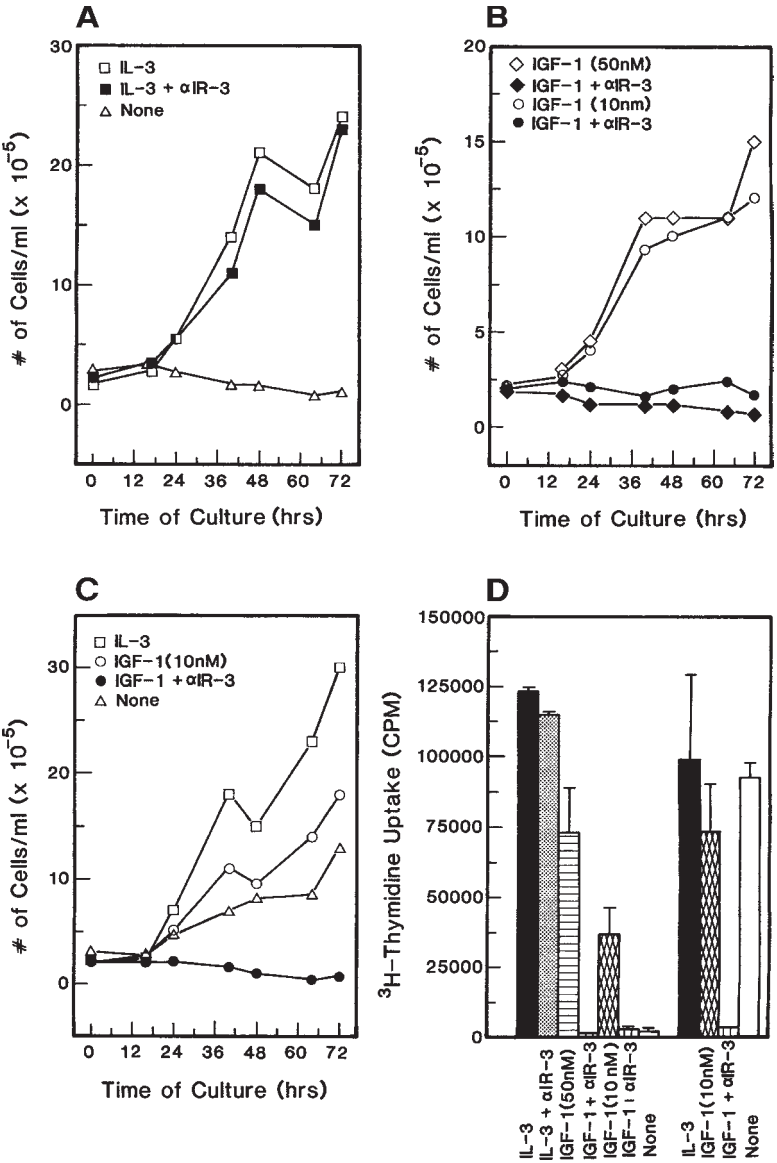

Fig. 9. Effects of hIGF-IR on Cell Growth. Cell growth was measured by counting the cells daily. Panel (**A**) FD/LISN (IGF-I + G418) cells cultured with: IL-3 (□), IL-3 + the αIGF-IR Ab (αIR-3) (■), or neither IL-3 nor αIGF-IR Ab (△). Panel (**B**) FD/LISN (IGF-I +G418) cl.1 cells cultured with: 50 nM IGF-I, (◇) IGF-I + αIGF-IR Ab (◆), 10 nM IGF-I (○), or 10 nM IGF-I + αIGF-IR(●). Panel (**C**) FD/LISN (DMEM + BCS)cl. 1 cells cultured with: IL-3 (□) 10 nM IGF-I (○), 10 nM IGF-I plus αIGF-IR (●) or no additional supplement (DMEM + BCS) (●) Panel (**D**) ^{3}H-Thymidine incorporation was determined after 24 h in the indicated culture conditions.

We have determined that the cells infected with the *WT Raf*-1 oncogene, which remain cytokine-dependent, do not readily form tumors in mice. Whereas the cells injected with the *CA Raf*-1 oncogene readily form tumors upon injection into immunocompromised mice. Thus the activated *Raf*-1 oncogene can render certain hematopoietic cells malignant ***(8)***.

13. A disadvantage of using *WT* and *CA Raf* constructs is that *Raf* activity cannot be manipulated in the way that *Cond Raf* constructs allow. Moreover, infection of factor independent cells with *WT* or *CA Raf* retroviral constructs can be problematic because of the difficulty in selecting for these cells. Expression of introduced genes should be confirmed in all cells, but is particularly important for factor independent cells that may be more resistant to selection. Clones and pools can be analyzed for expression and inheritance of the *Raf* genes by Western blot analysis, RT-PCR, and Southern blot analysis. We have found that many cells infected with a retrovirus containing pLNC failed to express *Raf*, but did express *neo*^{r}. This difference may result from differences in the promotor sequences driving Raf and *neo*^{r} transcription, which are CMV and Mo-MuLV-LTR sequences, respectively. In many of our studies with hematopoietic cells, we selected for cells that grew in response to WT or CA Raf expression. In these clones, inheritance and expression of the *Raf* gene was demonstrated by Southern and Western blot analysis. Another disadvantage of the *WT* and *CA* genes is that one cannot definitively demonstrate that the *Raf* gene is the only gene required for transformation because it is impossible to determine whether cells infected with WT or CA *Raf* revert to a nontransformed state by shutting off Raf activity. Other mechanisms could account for abrogation of cytokine dependence, such as insertion of the provirus into the vicinity of another gene that is also necessary for transformation. This concept is overcome, in part, by Cond Raf constructs (see later). Moreover, addition of GFP to WT and CA Raf constructs allow investigators a means to identify whether a cell is transfected, how much protein cells express, and also provides a tag which allows the protein to be purified from endogenous cellular proteins by antibodies to GFP that are now commercially available.
14. Dr. McMahon and colleagues have constructed a series of *Cond Raf* genes by fusing in frame the CR1 and CR2 deleted Raf-1, A-Raf and B-Raf cDNAs to the hb domain of the estrogen receptor. With these Cond constructs, the role of Raf in abrogation of cytokine dependence of hematopoietic cells or the induction of drug resistance in breast cancer cells can be determined in the absence of constitutive Raf activity. If a particular Raf gene is necessary for transformation or induction of drug resistance, these phenotypes should be reversed by hormone removal. With these constructs we have determined that the *Cond Raf* genes will abrogate the cytokine dependence of certain but not all hematopoietic cells ***(8,9,14)*** and induce drug resistance in breast cancer cells ***(40)***.
15. The use of CA oncogenes or other proteins to investigate signal transduction pathways is often difficult to interpret because the cells may be transformed and there may be many interacting pathways which are activated that have little direct interaction with the activated oncogene that has been introduced into the cell. This may be

overcome in part by transfection of *DN* genes corresponding to the activated oncogene and then determining whether the particular pathway was affected. Due to the interaction between signal transduction pathways and the frequent production of stimulatory growth factors, often only global changes can be addressed by transfection of a *CA* oncogene, i.e., does the oncogene result in the malignant transformation of the cells? Does the oncogene result in an increase in the resistance of the cells to a particular chemotherapeutic drug? These questions can be important and sometimes best answered with CA oncogenes. However, *Cond* oncogenes are sometimes the best answer to get around this problem. As discussed earlier, certain *Cond* oncogenes can be regulated by temperature (e.g., v-*src*^{ts}, v-*abl*^{ts}) and hormones (estrogen, testosterone, and progesterone). The advantage of these *Cond* oncogene mutant constructs is that they can be turned on and off by modifying the culture conditions. Then the effects of the oncogene on various signal transduction, cell cycle regulatory and apoptotic pathways can be identified. Thus one can determine whether the induction of the *Cond* gene results in the induction or suppression of the particular gene of interest. An example of how these conditional oncogenes can be used to investigate signal transduction pathways have been done with the ΔRaf-1:ER constructs which we have introduced into IL-3 dependent cells. We have observed that the Cond ΔRaf:ER genes will induce the activation of the PI3K pathway. We have shown this by the use of constructs, which will respond to 4HT. We have used cytokine-dependent GFPΔRaf-1:ER infected FL5.12 cells to determine that activation of GFPΔRaf-1:ER will result in the rapid activation of the PI3K pathway. This was determined by performing PI3K assays with αphosphotyrosine precipitated proteins and performing PI3K assays followed by resolution of the phospholipid species by thin layer chromatography. These cytokine-dependent GFPΔRaf-1:ER infected FL5.12 cells, which express low levels of the GFPΔRaf-1:ER protein as determined by Western blot analysis with an αER Ab and by flow cytometric analysis do not express autocrine cytokine mRNA transcripts. Thus, activation of the PI3K pathway is not likely due to abnormally high levels of the GFPΔRaf-1:ER protein or expression of autocrine cytokines. Furthermore, activation of this Raf pathway results in phosphorylation of the pro-apoptotic Bad protein. This was determined by radiolabeling cytokine and 4HT deprived cells with inorganic phosphate, then treating with IL-3, 4HT or ethanol (the vehicle for the 4HT) for 2 h. Bad immunoprecipitated from these cells was found to be phosphorylated. An advantage of the *Cond* system in cytokine-dependent cells is that the effects of the oncogene in the absence of selection for transformation can be determined. Furthermore, these cells may not express as much of the introduced GFPΔRaf-1:ER protein as those clones that were selected for factor-independence. Thus, this factor-dependent system may provide clues as to which pathways are induced by the activated Raf oncoprotein.

There are two other advantages of the GFPΔRaf-1:ER oncoprotein. First, the GFPΔRaf-1:ER oncoprotein has been modified (GFPΔRaf-1:ER*) so that it responds 100-fold more efficiently to 4HT than β-estradiol ***(30,32)***. This becomes important because some hormones such as β-estradiol have been shown to induce PI3K

activity in breast cancer cells ***(52)***. Second, GFP and ER tags allow the investigator to immunoprecipitate proteins that bind to the GFPΔRaf-1:ER protein with αGFP and αER Abs without interfering with proteins that may be bound to ΔRaf-1. Thus, if an investigator wants to find out which proteins bind to GFPΔRaf-1:ER, they can be pulled down with αGFP and αER Abs. The cells will have an endogenous ER protein, which is recognized by the ER Ab, but they should not have an endogenous protein, which is recognized by the αGFP protein.

36. Apoptotic pathways can also be investigated with *Cond Raf* and *Akt* genes. While these proteins and the effected downstream proteins can be studied by western blotting analysis with phosphospecific antibodies. Another aspect of apoptosis is more global, what are the morphological and DNA fragmentation events, which may be blocked by these pathways? We have determined that hematopoietic cells such as FL5.12 cells are not transformed by either Cond ΔRaf-1:AR or ΔAkt:ER* by themselves. However, inheritance of both ΔRaf-1:AR and ΔAkt:ER* will result in the conditional transformation of some of the cells. These cells require testosterone to activate the ΔRaf-1:AR and 4HT to activate the ΔAkt:ER* in order for proliferation to occur. In these cells the activation of the Akt activity by Raf activation alone is not sufficient to promote a strong enough anti-apoptotic response whereas activation of both ΔRaf-1AR and ΔAkt:ER* prevents apoptosis. This allows us the ability to investigate which events in apoptosis are prevented by either pathway alone or both pathways together. Activation of either Raf or Akt pathways by themselves hinders some early apoptotic events as determined by annexin V/PI binding, whereas activation of Raf by itself does not inhibit the late events in apoptosis as determined by TUNEL assays. Thus with these cells, it will be possible to identify some of the effects on apoptosis induced by the Raf and Akt pathways.
37. The effects of the ΔRaf-1:ER, ΔA-Raf:ER and ΔB-Raf:ER proteins on cell cycle progression in Raf-responsive hematopoietic cells have also been examined ***(53)***. The Raf proteins have been observed to induce the expression of $p21^{Cip1}$ but repress the expression of $p27^{Kip1}$. Furthermore, the Raf proteins induced the expression of the *c-myc* and *cyclin* genes and cdk2 kinase activity. A problem with these studies is that these Raf responsive cells will also express autocrine cytokines, which complicates the issue.
38. It is important to be aware that some phosphospecific antibodies actually measure inactivation of a protein. For example, a phosphospecific Ab sold by Cell Signaling recognizes Raf-1 when it is phosphorylated on S259, which is usually associated with inactivation of the protein as opposed to activation.
39. A goal of unraveling signal transduction pathways may be to identify potential sites which may be targets for therapy by small molecular weight drugs developed by the pharmaceutical industry, academic institutions, or governmental agencies. Moreover, effects of these inhibitors on Cond transformed cells is particularly useful because effects of the drug on "normal" and "transformed" cells can be determined within the same cell. We have used Cond transformed FD/ΔRaf-1:ER and FD/ΔA-Raf:ER hematopoietic cells to determine that the Cond transformed cells

may be more sensitive to various inhibitors (e.g., Raf, PI3K inhibitors) than the normal cells. This may be the result of the Cond transformed cells growing in response to the activated oncogene and other pathways are not maximally stimulated as occurs in the cytokine treated cell.

The specificity of certain drugs can be evaluated with the Cond transformed cells as we have determined that the FD/ΔRaf-1:ER and FDΔA-Raf:ER cells are sensitive to a Raf inhibitor where as the FD/ΔB-Raf:ER cells are not. This may result from the interaction of the Raf inhibitor with the kinase domain present in ΔRaf-1:ER and ΔA-Raf:ER but not the ΔB-Raf:ER.

Furthermore, the interactions between various signal transduction pathways can be determined by the combination of specific inhibitors and Cond oncogenes. The impingements of one pathway on another pathway and the requirement of these interactions can be investigated. For example, is the activation of the PI3K pathway by Raf dependent upon MEK1 activity or is it MEK1 independent? This question can be addressed by treatment of FL/GFPΔRaf-1:ER cells with the MEK1 inhibitors PD98059 or U0126 and then stimulate the cells and allow the inhibitors to exert their effects by incubation for 1 to 2 h with 4HT to activate Raf and then performing PI3K assays and determine whether PI3K activity induced by Raf does or does not require MEK1 activity. The interactions between signal transduction pathways can be identified and elucidated by Cond oncoproteins.

40. The involvement of an oncogene in the induction of resistance to chemotherapeutic drugs can be examined by infecting target cells with a particular oncogene and then determining whether the overexpression of the oncogene confers resistance to the chemotherapeutic drug. The use of *Cond* oncogenes may in some cases be preferred to *CA* oncogene as the effects of the oncogene on drug resistance can be controlled by addition or removal of the agent, which regulates the activity of the oncogene. For example we have infected MCF-7 breast cancer cells with a conditional form of the *Raf*-1 oncogene (ΔRaf-1:AR) ***(40–42)***. The ability of Raf to induce the drug resistance of MCF-7 was determined by culturing the cells in the presence and absence of testosterone. We found that activation of Raf-1 promoted MCF-7 cell drug resistance that was mediated, at least in part, by MDR-1, (a.k.a P-glycoprotein pump, P-gp), *Bcl*-2, and autocrine growth factor expression ***(40–42)***. Whether elevated expression of these proteins is necessary for MCF-7 cell drug resistance is being determined.

We have also introduced CA oncogenes into MCF-7 cells and determined their effects on drug resistance ***(40–42)***. Overexpression of CA v-Ha-Ras, Raf, and Bcl-2 also increased the drug resistance of MCF-7 cells. However, overexpression of *Bcl-2* did not result in an increase in MDR-1 expression indicating that Raf may induce additional molecules, which contribute to a drug resistant phenotype ***(42)***. Furthermore, addition of a *Cond Raf*-1 molecule synergized with *Bcl*-2 overexpression to increase the ability of the cells to grow in the presence of chemotherapeutic drugs. *Raf*-1 and *Bcl*-2 expression resulted in the increased resistance of the cells to grow in the presence of doxorubicin and *Raf*-1 expression also decreased the sensitivity of the cells to paclitaxel, another substrate for the MDR-1. However, although *Bcl*-2 did increase the resistance of the cells to doxorubicin, it did

not increase their resistance to paclitaxel indicating that the two different oncogenes had some different effects on the induction of drug resistance.

In summary, this chapter has described how retroviral vectors can be used to study signal transduction, apoptotic and drug resistant pathways. We have discussed the advantages and disadvantages of certain models with the goal of providing the reader with an idea of what they will encounter with these methods of gene transfer.

References

1. Weiss, R., Teich, N., Varmus, H., and Coffin, J. (1984) *RNA Tumor Viruses*. Cold Spring Harbor Laboratory Press, 2nd ed.
2. Miller, A. D. and Rosman, G. J. (1989) Improved retroviral vectors for gene transfer and expression. *Biotechniques* **7,** 980–990.
3. Shimada, T., Fujii, H., Mitsuya, H., and Nienhuis, A. W. (1991) Targeted and highly efficient gene transfer into CD4+ cells by a recombinant human immunodeficiency virus retroviral vector. *J. Clin. Inves.* **88,** 1043–1047.
4. Mulligan, R. C. and Berg, P. (1981) Selection for animal cells that express the Escherichia coli gene encoding for xanthine-guanine phosphoribosyltransferase. *Proc. Natl. Acad. Sci. USA* **78,** 2072–2076.
5. McCubrey, J. A., Lee, J. T., Steelman, L. S., Blalock, W. L., Moye, P. W., Chang, F., et al. (2001) Interactions between the PI3K and Raf signaling pathways can result in the transformation of hematopoietic cells. *Cancer Detect. Prevent.* **25,** 375–393.
6. McCubrey, J. A., Smith, S. R., Algate, P. A., DeVente, J. E., White, M. K., and Steelman, L. S. (1993) Retroviral infection can abrogate the factor-dependency of hematopoietic cells by autocrine and non-autocrine mechanisms depending on the presence of a functional viral oncogene. *Oncogene* **8,** 2905–2915.
7. McCubrey, J., Steelman, L. S., Wang, X.-Y., Davidian, E. W., Hoyle, P. E., White, C., et al. (1995) Autocrine growth factor secretion after transformation of human cytokine-dependent cells by viral and cellular oncogenes. *Int. J. Oncol.* **7,** 573–586.
8. Hoyle, P. E., Moye, P. W., Steelman, L. S., Blalock, W. L., Franklin, R. A., Pearce, M., et al. (2000) Differential abilities of the Raf family of protein kinases to abrogate cytokine-dependency and prevent apoptosis in murine hematopoietic cells by a MEK1-dependent mechanism. *Leukemia* **14,** 642–656.
9. Moye, P. W., Blalock, W. L., Hoyle, P. E., Chang, F., Franklin, R. A., Weinstein-Oppenheimer, C., et al. (2000) Synergy between Raf and BCL2 in abrogating the cytokine dependency of hematopoietic cells. *Leukemia* **14,** 1060–1079.
10. Blalock, W. L., Moye, P. W., Chang, F., Pearce, M., Steelman, L. S., McMahon, M., and McCubrey, J.A. (2000) Combined effects of aberrant MEK1 activity and BCL2 overexpression on relieving the cytokine-dependency of human and murine hematopoietic cells. *Leukemia* **14,** 1080–1096.
11. Blalock, W. L., Moye, P. W., Steelman, L. S., Franklin, R. A., McCarthy, S. A., Cherwinski, H., et al. (2000) A conditionally-active form of MEK1 results in autocrine transformation of human and mouse hematopoietic cells. *Oncogene* **19,** 526–536.

12. Blalock, W. L., Moye, P. W., Chang, F., Lee, J. T., Pohnert, S. C., Burrows, C., et al. (2001) Effects of inducible MEK1 activation on the cytokine dependency of lymphoid cells. *Leukemia* **15,** 794–807.
13. McCubrey, J. A., Steelman, L. S., Wang, X.-Y., Algate, P. A., Hoyle, P. E., White, C., et al. (1995) Differential effects of viral and cellular oncogenes on the growth factor-dependency of hematopoietic cells. *Int. J. Oncol.* **7,** 295–310.
14. McCubrey, J. A., Steelman, L. S., Hoyle, P. E., Blalock, W. L., Weinstein-Oppenheimer, C., Franklin R. A., et al. (1998) Differential abilities of activated Raf oncoproteins to abrogate cytokine-dependency, prevent apoptosis and induce autocrine growth factor synthesis in human hematopoietic cells. *Leukemia* **12,** 1903–1929.
15. Weinstein-Oppenheimer, C., Steelman, L. S., Algate, P. A., Blalock, W. L., Burrows, C., Hoyle, P. E., et al. (2000) Effects of deregulated Raf activation on integrin, cytokine-receptor expression and the induction of apoptosis in hematopoietic cells. *Leukemia* **14,** 1921–1938.
16. White, M. K. and McCubrey, J. A. (2001) Suppression of apoptosis: role in cell growth and neoplasia. *Leukemia* **15,** 1011–1121.
17. McCubrey, J. (1985) DNA-mediated transformation of lymphoid cells, in *Immunological Methods.* Vol. III, Orlando Academic, Orlando, FL, pp. 21–29.
18. Daley, G. Q., Van Etten, R. A., and Baltimore, D. (1990) Induction of chronic myelogenous leukemia in mice by the P210bcr/abl gene of the Philadelphia chromosome. *Science* **247,** 824–830.
19. Wong, P. M., Chung, S. W., and Nienhuis, A. W. (1987) Retroviral transfer and expression of the interleukin-3 gene in hemopoietic cells. *Genes Dev.* **1,** 358–365.
20. McCubrey, J. A., Steelman, L. S. Mayo, M. W., Algate, P. A., Dellow, R. A., and Kaleko, M. (1991) Growth promoting effects of insulin-like growth factor-1 on hematopoietic cells: overexpression of introduced IGF-I receptor abrogates IL-3-dependency of murine factor dependent cells by a ligand-dependent mechanism. *Blood* **78,** 921–929.
21. White, M. K., DeVente, J. E., Robbins, P. J., Canupp, D. M., Mayo, M. W., Steelman, L. S., and McCubrey, J. A. (1994) Differential regulation of glucose transporter expression in hematopoietic cells by oncogenic transformation and cytokine stimulation. *Oncol. Rep.* **1,** 17–26.
22. White, M. K. and McCubrey, J. A. (1995) Changes in glucose transport associated with malignant transformation. *Int. J. Oncol.* **7,** 701–712.
23. Poste, G. and Flood, M. K. (1979) Cells transformed by temperature-sensitive mutants of avian sarcoma virus cause tumors in vivo at permissive and nonpermissive temperatures. *Cell* **17,** 789–800.
24. Kipreos, E. T. and Wang, J. Y. (1988) Reversible dependence on growth factor interleukin-3 in myeloid cells expressing temperature sensitive v-abl oncogene. *Oncogene Res.* **2,** 277–284.
25. Daley, G. Q., McLaughlin, J., Witte, O. N., and Baltimore, D. (1987) The CML-specific P210 bcr/abl protein, unlike v-abl, does not transform NIH/3T3 fibroblasts. *Science* **237,** 532–535.

26. Kohler, G., Baumann, B., Iglesias, A., McCubrey, J., Potash, M. J., Traunecker, A., and Zhu, D. (1984) Different ways to modify monoclonal antibodies. *Med. Oncol. Tumor Pharmacother.* **1,** 227–233.
27. McCubrey, J., McKearn, J. P., and Kohler, G. (1985) Transformation of B and non-B cell lines with the 2,4,6,-trinitrophenyl (TNP)-specific immunoglobulin genes. *Eur. J. Immunol.* **15,** 1117–1124.
28. Eilers, M., Picard, D., Yamamoto, K. R., and Bishop, J. M. (1989) Chimaeras of myc oncoprotein and steroid receptors cause hormone-dependent transformation of cells. *Nature* **340,** 66–68.
29. Samuels, M. L., Weber, M. J., Bishop, J. M., and McMahon, M. (1993) Conditional transformation of cells and rapid activation of the mitogen-activated protein kinase cascade by an estradiol-dependent human raf-1 protein kinase. *Mol. Cell. Biol.* **13,** 6241–6252.
30. Woods, D., Parry, D., Cherwinski, H., Bosch, E., Lees, E., and McMahon, M. (1997) Raf-induced proliferation or cell cycle arrest is determined by the level of Raf activity with arrest mediated by p21Cip1. *Mol. Cell. Biol.* **17,** 5598–5611.
31. Mirza, A. M., Kohn, A. D., Roth, R. A., and McMahon, M. (2000) Oncogenic transformation of cells by a conditionally active form of the protein kinase Akt/PKB. *Cell Growth Differ.* **11,** 279–292.
32. McMahon, M. (2001) Steroid receptor fusion proteins for conditional activation of Raf-MEK-ERK signaling pathway. *Meth. Enzymol.* **332,** 401–4117.
33. Wunderlich, F. T., Wildner, H., Rajewsky, K., and Edenhofer, F. (2001) New variants of inducible Cre recombinase: a novel mutant of Cre-PR fusion protein exhibits enhanced sensitivity and an expanded range of inducibility. *Nucleic Acids Res.* **29,** E47.
34. Peyrollier, K., Hajduch, E., Gray, A., Litherland, G. J., Prescott, A. R., Leslie, N. R., and Hundal, H. S. (2000) A role for the actin cytoskeleton in the hormonal and growth-factor-mediated activation of protein kinase B. *Biochem. J.* **352,** 617–622.
35. Wang, X.-Y. and McCubrey, J. A. (1997) Regulation of interleukin-3 expression in normal and autocrine transformed hematopoietic cells. *Int. J. Oncol.* **10,** 989–1001.
36. Lloyd, A. C., Obermuller, F., Staddon, S., and Barth, C. F., McMahon M., and Land, H. (1997) Cooperating oncogenes converge to regulate cyclin/cdk complexes. *Genes Dev.* **11,** 663–677.
37. Pritchard, C. A., Samuels, M. L., Bosch, E., and McMahon, M. (1995) Conditionally oncogenic forms of the A-Raf and B-Raf protein kinases display different biological and biochemical properties in NIH 3T3 cells. *Mol. Cell. Biol.* **15,** 6430–6442.
38. Bosch, E., Cherwinski, H., Peterson, D., and McMahon, M. (1997) Mutations of critical amino acids affect the biological and biochemical properties of oncogenic A-Raf and Raf-1. *Oncogene* **15,** 1021–1033.
39. McCarthy, S. A., Samuels, M. L., Pritchard, C. A., Abraham, J. A., and McMahon, M. (1995) Rapid induction of heparin-binding epidermal growth factor/diphtheria toxin receptor expression by Raf and Ras oncogenes. *Genes Dev.* **9,** 1953–1964.
40. Weinstein-Oppenheimer, C. R., Henriquez-Roldan, C. F., Davis, J. M., Navolanic, P. M., Saleh, O. A., Steelman, L. S., et al. (2001) Role of the Raf signal transduc-

tion cascade in the *in vitro* resistance to the anticancer drug doxorubicin. *Clin. Cancer Res.* **7,** 2898–2907.
41. Weinstein-Oppenheimer, C. R., Burrows, C., Steelman, L. S., and McCubrey, J. A. (2002) The effects of β-estradiol on Raf activity, cell cycle progression and growth factor synthesis in the MCF-7 breast cancer cell line. *Cancer Biol. Ther.* **1,** 254–260.
42. Davis, J. M., Weinstein-Oppenheimer, C. R., Steelman, L. S., Navolanic, P. M., Hu, W., Konopleva, M., et al. (2003) Raf-1 and Bcl-2 induce distinct and common pathways which contribute to breast cancer drug resistance. *Clin. Cancer Res.*, in press.
43. Steelman, L. S., Algate, P. A., Blalock, W. L., Wang, X.-Y., Prevost, K. D., Hoyle, P. E., and McCubrey, J. A. (1996) Oncogenic effects of overexpression of the interleukin-3 receptor on hematopoietic cells. *Leukemia* **10,** 528–542.
44. Blalock, W. L., Weinstein-Oppenheimer, C., Chang, F., Hoyle, P. E., Wang, X. Y., Algate, P. A., et al. (1999) Signal transduction, cell cycle regulatory, and anti-apoptotic pathways regulated by IL-3 in hematopoietic cells: possible sites for intervention with anti-neoplastic drugs. *Leukemia* **13,** 1109–1166.
45. McCubrey, J. A., May, W. S., Duronio, V., and Mufson, A. (2000) Serine/threonine phosphorylation in cytokine signal transduction. *Leukemia* **14,** 9–21.
46. Wang, X. Y., Steelman, L. S., and McCubrey, J. A. (1997) Abnormal activation of cytokine gene expression by intracisternal type A particle transposition: effects of mutations which result in autocrine growth factor stimulation and malignant transformation. *Cytokines Cell Mol. Ther.* **3,** 3–19.
47. McCubrey, J. A., Blalock, W. L., Chang, F., Steelman, L. S., Pohnert, S. C., Navolanic, P. M., et al. (2001) Signal transduction pathways: cytokine model, in *Cell Cycle Checkpoints and Cancer*, *Molecular Biology Intelligence Unit 15*, (Blagosklonny, M. V., ed.), Landes Bioscience, Austin, TX, pp. 17–51.
48. Lee, J. T. Jr. and McCubrey J. A. (2002) The Raf/MEK/ERK signal transduction cascade as a target for chemotherapeutic intervention in leukemia. *Leukemia* **16, i**n press.
49. Weinstein-Oppenheimer, C. R., Blalock, W. L., Steelman, L. S., Chang, F., and McCubrey, J. A. (2000) Raf signal transduction cascade as a target for chemotherapeutic intervention in growth factor-dependent tumors *Pharmacol. Ther.* **88,** 229–279.
50. Fletcher, B. S., Dragstedt, C., Notterpek, L., and Nolan, G. P. (2002) Functional cloning of Spin-2, a nuclear anti-apoptotic protein with roles in cell cycle progression. *Leukemia,* in press.
51. Kaleko, M., Rutter, W. J., and Miller, A. D. (1990) Overexpression of the human insulinlike growth factor I receptor promotes ligand-dependent neoplastic transformation. *Mol. Cell. Biol.* **10,** 464–473.
52. Sun, M., Paciaga, J. E., Feldman, R. I., Yuan, Z. Q., Coppola, D., Lu, Y. Y., et al. (2001) Phosphatidylinositol-3-OH kinase (PI3K)/AKT2, activated in breast cancer regulates and is induced by estrogen receptor α (ERα) via interaction between ERα and PI3K. *Cancer Res.* **61,** 5985–5991.
53. Chang, F. and McCubrey, J. A. (2001) $p21^{Cip1}$ induced by Raf is associated with increased Cdk4 activity in hematopoietic cells. *Oncogene* **20,** 4353–4364.

III

Protein Interactions

18

Methods for the Study of Protein–Protein Interactions in Cancer Cell Biology

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Abstract

Development of sensitive methods to monitor and quantitatively assess the expression levels of endogenous genes and the association-interaction of proteins in living cells and whole organisms is a complex and challenging problem. In this chapter, we have described basic methods for investigating protein–protein interactions which include immunoprecipitation, GST pull-down assays, peptide bead pull-down assays, chemical crosslinking and photoaffinity labeling. These methods should provide important tools to dissect crosstalk between proteins and the direct implications of this crosstalk in signaling pathways and cancer biology.

Key Words: Signaling; association; direct-interaction; protein–protein interactions; immunoprecipitation; GST-binding domains.

1. Introduction

A major component of the biochemistry of cancer cell signaling is related to changes in the interactions of various proteins in response to growth factors, cytokines, and hormones. Other protein–protein interactions are not signal dependent, but are important in maintaining the cancer cell phenotype. In this chapter, we have described in detail some basic procedures for investigating protein–protein interactions that will be helpful to the cancer biologist. These include immunoprecipitation, GST pull-down assay, peptide bead pull-down assay, chemical crosslinking, and photoaffinity labeling. Often the interactions to be evaluated are phosphorylation-dependent. Because the interactions of peptide

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hormones with their receptors are also protein–protein interactions, we have included a method for determination of the binding of peptide hormones to plasma membrane receptors. These methods will provide a means for the investigator to analyze the importance of particular protein–protein interactions in signaling pathways leading to the tumorigenic phenomena such as increased growth, increased cell survival, changes in gene expression, angiogenesis, and metastasis.

2. Materials

2.1. Immunoprecipitation and Analysis of Associated Proteins

1. RIPA buffer: 50 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl, 1% NP-40, 0.5% Deoxycholate, 0.1% sodium dodecyl sulfate (SDS).
2. NP40 Lysis buffer: 50 m*M* Tris-HCl, pH 8.0, 150 m*M* NaCl, 1% NP-40.
3. Triton X-100. Lysis Buffer: 50 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl, 1% Triton X-100. All lysis buffers should include a broad spectrum of protease inhibitors. A good choice is the Complete™ protease inhibitors from Roche, Inc. (Indianapolis, IN). In the case that tyrosine phosphorylation is critical to the associations, 1 m*M* sodium orthovanadate should also be included in the buffer (*see* **Note 1**). If serine and threonine phosphorylations are critical to the association, 50 m*M* β-glycerol phosphate should be included.
4. Bio-Rad Protein Assay (Bio-Rad, Hercules, CA).
5. Protein G and Protein A Sepharose (Pierce Chemical Co., Rockford, IL).
6. Control IgGs (Santa Cruz Biotechnology, Santa Cruz, CA).
7. Trans^{35}S-Label ^{35}S methionine/^{35}S cysteine mixture (ICN, Costa Mesa, CA).
8. SDS sample buffer (3X stock): 50 m*M* Tris-HCl, pH 6.8, 0.45 *M* dithiothreitol (DTT), 30% sucrose, 6% SDS, 0.025% bromophenol blue.
9. Transfer buffer: 25 m*M* Tris, 193 m*M* glycine, 20% methanol, 0.1% SDS.
10. Phosphate-buffered saline (PBS): Premixed powder (Sigma Chemical Co., St. Louis, MO).
11. Tris-buffered saline (TBS): 25 m*M* Tris-HCl, pH 7.4, 137 m*M* NaCl, 2.7 m*M* KCl.
12. PBST (Phosphate-buffered saline-Tween): supplemented with 0.1% Tween-20.
13. TBST (Tris-buffered saline-Tween): supplemented with 0.1% Tween-20.
14. BSA Blocking Buffer: 4% bovine serum albumin (BSA) dissolved in PBST, supplemented with 1 m*M* sodium azide, and filtered through a 0.45-μm filter.
15. Milk Blocking Buffer: 5% nonfat dried milk dissolved in PBST.
16. ECL Chemiluminescence reagent (Amersham Biosciences, Piscataway, NJ).

2.2. Association of Proteins to GST Fusion Proteins by GST Pull-Down Assay

1. GST Lysis buffer: 20 m*M* Tris-HCl, pH 7.4, 137 m*M* NaCl, 1 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, 1% NP-40, 1 m*M* Na_3VO_4.
2. Glutathione Sepharose 4B (Amersham Biosciences).

3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) reagents (*see* **Subheading 2.1., steps 8–9).**

2.3. Peptides Linked to Beads Used in Pull-Down Assays

1. Affi-Gel 15 (Bio-Rad).
2. Association buffer 20 m*M* Tris-HCl, pH 7.4, 137 m*M* NaCl, 1 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, 1% NP-40, 1 m*M* Na_3VO_4.
3. SDS-PAGE reagents (*see* **Subheading 2.1., steps 8–9).**

2.4. Chemical Crosslinking of Associated Proteins

1. Dithiobis [succinimidyl propionate] (DSP) (Pierce Chemical Co., Rockford, IL).
2. Stopping buffer: 1 *M* Tris-HCl, pH 7.4.
3. Nonreducing SDS sample buffer: 6% SDS, 30% glycerol, 50 m*M* Tris-HCl, pH 6.8, 0.025% bromophenol blue.
4. SDS-PAGE reagents (*see* **Subheading 2.1., steps 8–9**).

2.5. Photoaffinity Labeling of Associated Proteins

1. IODO-GEN precoated tubes (cat. #28601, Pierce Chemical Co., Rockford, IL).
2. Sulfosuccinimidyl 2-(p-azidosalicylamido) ethyl-1-3'-dithiopropionate (SASD) (Pierce Chemical Co.).
3. ^{125}I Sodium Iodide, carrier free (Perkin-Elmer Life Sciences, Boston, MA).
4. Biospin P6 columns (Bio-Rad).
5. SDS-PAGE reagents (*see* **Subheading 2.1., steps 8–9**).

2.6. Plasma Membrane Receptor-Ligand Binding Determination

1. IODO-GEN precoated tubes (cat. #28601, Pierce Chemical Co.).
2. ^{125}I Sodium Iodide, carrier free (Perkin-Elmer Life Sciences).
3. Scavenger buffer: 10 mg/mL L-Tyrosine in PBS.
4. Tris Iodination buffer: 25 m*M* Tris-HCl, pH 7.5, 0.4 *M* NaCl.
5. Tris/BSA buffer: 0.25% bovine serum albumin, 25 m*M* Tris-HCl, pH 7.5, 0.4 *M* NaCl, 5 m*M* ethylenediamine tetraacetic acid (EDTA), 0.05% sodium azide.
6. Biospin P6 columns (Bio-Rad).
7. Binding buffer: 20 m*M* MOPS, pH 7.4, 2 m*M* $MgCl_2$, 140 m*M* NaCl, 0.2% gelatin.
8. Lysis buffer: 0.1 *M* NaOH, 1% SDS.
9. SDS-PAGE reagents (*see* **Subheading 2.1., steps 8–9).**
10. 24-well tissue culture plates (Costar, Cambridge, MA).
11. Polystyrene gamma counter vials (Fisher Scientific, Norcross, GA).

3. Methods

3.1. Immunoprecipitation and Analysis of Associated Proteins

Immunoprecipitation for the purpose of observing associated proteins can be done in three different ways. One way to visualize the associated proteins is

after metabolic labeling of the cells, and another is by subjecting the precipitated proteins to western immunoblotting. A third way in which immunoprecipitates may be used for the purpose of studying protein–protein interactions involves a proteomics approach. For this purpose, we and others employ 2-D electrophoresis followed by MALD1 mass spectrometry to identify candidate proteins in the immunoprecipitates. The advantage of the former technique is that all the precipitated proteins are visualized, however, only the molecular weights of the proteins are revealed. In the latter technique, only selected proteins are revealed, depending on the blotting antibody, but the associating proteins can be determined depending on the specificity of the blotting antibodies.

1. Metabolic labeling of cells is most frequently carried out with [^{35}S] methionine, or mixtures of [^{35}S] methionine and [^{35}S] cysteine. For labeling, carrier free [^{35}S] methionine or [^{35}S] methionine + cysteine is mixed with methionine and cysteine-free medium. Cells are washed with the deficient medium, and then incubated with the deficient medium plus the radionucleotides at approx 0.05–0.10 mCi/10^6 cells for 2–4 h at 37ºC in a CO_2 incubator. If the cells need to be starved for stimulation with a ligand, then this should be done at this time. Cells may also have to be starved prior to the labeling if additional starvation is needed. After ^{35}S labeling, the cells are washed with unlabeled medium, stimulated with the ligand if desired, and then washed with cold PBS prior to lysis. ^{35}S-containing medium should be disposed of according to standard radiation safety protocols.
2. Lysis of the washed cells can be done by a number of different lysis buffers, including RIPA buffer, NP-40 lysis buffer, and Triton X-100 lysis buffer (*see* **Subheading 2.,** for composition). Generally, less harsh buffers such as the NP40 lysis buffer will yield more associations, although more nonspecific associations may also occur. After scraping lysed attached cells or suspending a pellet of cells into the lysis buffer, the lysates are vortexed briefly and gently to disperse the cell material, and are kept on ice to prevent any proteolysis.
3. Lysates are then centrifuged at 16,000*g* in a microcentrifuge for 15 min at 5ºC. Supernatants are balanced for protein by Bio-Rad Protein Assay or other protein assay, again with care taken to dispose of radioactive material properly.
4. Supernatants should be pre-absorbed with normal serum or control antibody (*see* **Materials, Subheading 2.1., step 6**) to minimize nonspecific associations. These should be added to the lysates and allowed to incubate for approx 1 h at 5ºC with rocking. After this time, an approx 50 µL bead volume of protein G Sepharose or protein A Sepharose is added, and the incubation is continued for 15 min at 5ºC with rocking. Consult Harlow and Lane ***(1)*** for antibody isotype specificities for binding to protein A or protein G. Preabsorbed supernatants are then carefully transferred to new polypropylene tubes.
5. The appropriate specific antibody is then added to the absorbed supernatants at approx 1 µg antibody/mg of lysate protein (*see* **Note 2**). Control incubations should be planned at this point utilizing a relevant control serum or antibody (*see* **Materials,**

Subheading 2.1., step 6) in place of the specific antibody. Time of incubation with the antibody should be between 1.5 and 18 h at 5ºC with rocking. After this time either protein A or protein G Sepharose, depending on the isotype of the antibody, is added (approx 15 µL bead volume/mL of incubation). Consult Harlow and Lane ***(1)*** for antibody isotype specificities for binding to protein A or protein G. Incubation is continued for 20 min at 5ºC with rocking.

6. Precipitates are then washed with the lysis buffer (3X with 1 mL of lysis buffer with intermittent centrifugation). Precipitates should always be suspended in the fresh lysis buffer prior to centrifugation (*see* **Note 3**).
7. Following washing, the precipitates should be solubilized in SDS sample buffer and heated to 100ºC for 2 min prior to analysis on SDS-PAGE ***(2)***. Samples can be stored at –20ºC for a number of days prior to running the SDS-PAGE.
8. Transfer and developing of autorads: immunoreactive bands are visualized using ECL reagents (Amersham Pharmacia Biotech). The horseradish peroxidase conjugated mouse anti-IgG is added for 1 h.

Immunoprecipitation with detection by Western blotting follows a similar protocol as above, except that cells are only starved as needed, and incubation with ^{35}S-containing medium is omitted. Steps are as follows.

1. Confluent cells are starved and then stimulated with growth factors as required by the experiment. Cells are then washed with cold PBS and then scraped into cold lysis buffer. The lysis buffers utilized include RIPA buffer, NP40 lysis buffer, or Triton X-100 lysis buffer (*see* **Materials, Subheading 2.1., step 1**). As indicated above, the NP40 lysis buffer is less harsh in terms of removing specific associations, but may yield a higher background. Attached cells are scraped into the lysis buffer using a disposable cell scraper. Suspended cells can be pelleted prior to the addition of lysis buffer, or resuspended in medium at a high concentration and lysed by the addition of a concentrated lysis buffer. Lysates should be vortexed briefly and then kept on ice.
2. Lysates are then centrifuged at 16,000*g* in a microcentrifuge at 5ºC. Supernatants are then pipeted and kept on ice. Total protein level is measured by the Bio-Rad protein assay or other methods in order to balance the protein in each sample.
3. Supernatants are preabsorbed with a control antibody as described in the previous section.
4. Specific antibody is then added to the preabsorbed supernatants at approx1 µg antibody/1 mg of lysate protein. Again, as indicated in **Note 2**, it is important when using commercially prepared antibodies to consult the specifications for use. Also, as indicated earlier, the appropriate precipitation controls, including a nonspecific antibody in place of the specific antibody, should be planned. After addition of the antibodies, lysates are incubated for 1.5 to 18 h at 5ºC with rocking. After this time, add protein A or protein G Sepharose (15 µL bead vol/mL of lysate), again consulting Harlow and Lane ***(1)*** for the specific antibody isotype association to protein A or G.

5. Precipitates are then washed with the lysis buffer (3X with 1 mL of lysis buffer with intermittent centrifugation). Precipitates should always be suspended in the fresh lysis buffer prior to centrifugation (*see* **Note 3**).
6. Following washing, the precipitates should be solubilized in SDS sample buffer and heated to 100ºC for 2 min prior to analysis on SDS-PAGE. Samples can be stored at –20ºC for a number of days prior to running the SDS-PAGE.
7. Samples are analyzed on a typical reducing SDS-PAGE ***(2)***, using the appropriate percentage of gel and prestained standards to indicate the molecular weights. Proteins are transferred to PVDF or nitrocellulose membranes (Bio-Rad, Amersham) using the Transfer Buffer as described (*see* **Materials, Subheading 2.1., steps 9–16**). A number of either wet or semidry transfer apparatuses are available for this purpose.
8. Transfers are blocked for 1 h in either BSA blocking buffer or milk blocking buffer (*see* **Notes 4** and **5**). Transfers are then incubated with the primary antibody diluted to approx 1 ng/mL in the blocking buffer used above. Incubation can be for 1.5 h at room temperature, or alternately 18 h at 5ºC with rocking.
9. Transfers are then washed in PBS + 0.1% Tween-20 (PBST) 3X, 5 min each wash. Transfers are then incubated with secondary HRP-linked antibody specific for binding to the primary antibody (usually either antirabbit HRP or antimouse HRP), diluted in PBST at 100 ng/mL IgG.
10. Transfers are then washed 3X with PBST/TBST, 5 min each wash. The washed transfer is visualized by incubation in ECL reagent (Amersham) for 1 min. The transfers are quickly wrapped in a plastic cover sheet (*see* **Note 5**) with cellophane tape to prevent leakage of liquid, and the transfer is visualized by exposure to X-ray film. Times of exposure are usually on the order of 5–30 s. For variations in secondary antibodies and methods of detection, consult Harlow and Lane ***(1)***.

3.2. Association of Proteins to GST Fusion Proteins by GST Pull-Down Assay

Another means of determining in vitro protein–protein associations is to use a purified glutathione-S-transferase fusion protein linked to a protein of interest and to add this fusion protein to a cellular lysate. The fusion protein is then precipitated by means of association to glutathione-linked beads. The precipitates are treated similarly to immunoprecipitation (ip)/western blotting in that the sample is resolved on SDS-PAGE, and transferred to immunoprecipitation PVDF or nitrocellulose. The transfer membrane is then subjected to western immunoblotting, as described in the earlier procedure. This methodology was used to show the association of the c-kit receptor tyrosine kinase to the SH2 domain of the cytoplasmic tyrosine kinase CHK (3) as shown in **Fig. 1**. The procedure for preparing the GST fusion protein precipitate is as follows.

1. Confluent tissue culture cells are starved appropriately and treated with growth factor according to the specific protocol. Attached cells are washed with cold PBS, and then scraped into 1 mL of GST lysis buffer (*see* **Note 6**)/10-cm plate. Cells are

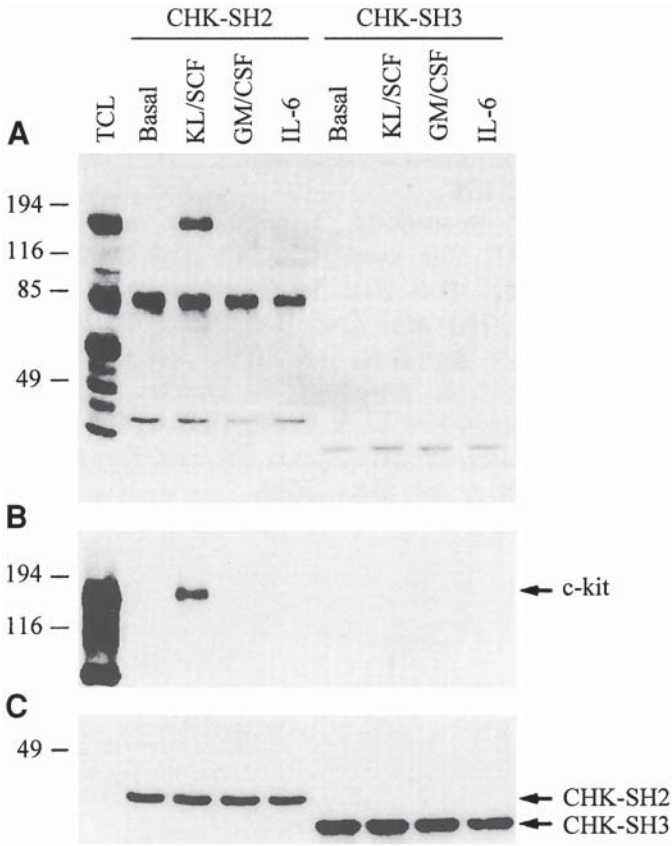

Fig. 1. Association of CHK-SH2 and CHK-SH3 with cellular phosphoproteins in stimulated CMK cells. The lysates were incubated with 10 μg of either CHK-SH2 or CHK-SH3 and glutathione sepharose. The washed precipitates were analyzed by SDS-PAGE followed by immunoblotting with either PY-20 (**A**), anti-c-Kit antibody (**B**), or anti-GST antibody (**C**).

vortexed gently and placed on ice. Suspended cells are either centrifuged and lysed as a pellet, or centrifuged, resuspended at a high concentration (10–50 μL) and lysed by the addition of a concentrated GST lysis buffer. Again, cells are vortexed gently, and placed on ice.

2. Lysates are centrifuged at 16,000*g* in a microcentrifuge for 15 min at 5ºC. The supernatants are pipetted into polypropylene tubes on ice. The protein concentrations are determined by the Bio-Rad protein assay method, or other methods.
3. To samples containing equal amounts of protein (approx 0.5–1.0 mg) is added either 10 μg of GST fusion protein, or GST protein without additional fusion protein as a control. The samples are incubated for 1.5 h at 5ºC with rocking.

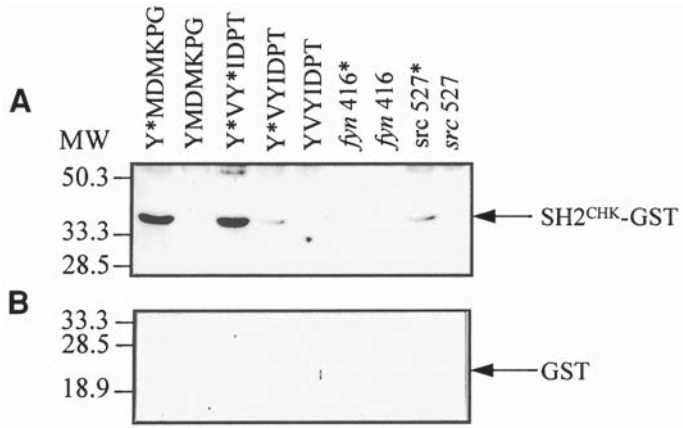

Fig. 2. Association of CHK-SH2 to phosphopeptides linked to beads. (**A**) CHK-SH2 GST fusion protein was incubated with phosphorylated and non-phosphorylated peptides linked to Affi-Gel 15 beads. Washed precipitates were run on SDS-PAGE and transfers were blotted with anti-GST antibody. (**B**) GST protein alone was incubated with the same phosphorylated and non-phosphorylated peptides linked to beads. As in panel **A**, washed precipitates were run on SDS-PAGE and transfers were blotted with anti-GST antibody.

4. Glutathione Sepharose is then added as a 50% bead volume suspension at approx 15 µL bead volume per 1 mL of sample. The sample is incubated for 30 min at 5ºC with rocking.
5. The precipitate is then washed 3X with 1 mL of lysis buffer with intermittent centrifugation. To the final precipitate is added 15 µL of SDS sample buffer. The sample is then heated to 100ºC for 2 min after which it can be stored at –20ºC prior to running SDS-PAGE.
6. Associated proteins are detected by SDS-PAGE followed by western immunoblotting, using the procedure described earlier.

3.3. Peptides Linked to Beads Used in Pull-Down Assays

Another means of showing a specific protein–protein interaction is by chemically linking a short peptide to a Sepharose bead. Incubations can then be carried out with either purified proteins, or crude extracts to show the specific association with certain sequences. This was done in our laboratory to show the association of particular phosphorylated sequences of the c-kit receptor to the Csk Homologous Kinase (CHK) SH2 domain ***(4)*** (*see* **Fig. 2**).

The method of preparing the peptide-linked beads is as follows.

1. Prepare the peptides by diluting to 1 µ*M* in a buffer not containing amino groups, such as 0.1 *M* MOPS, pH 7.5.

2. Wash 0.5 mL of bead volume of Affigel-15 three times with 25 mL in cold deionized H_2O, and aspirate the supernatant, keeping the beads on ice.
3. Add 1 μ*M* of peptide to the beads and incubate 1.5 h with rocking at room temperature.
4. Add 50 μL of 1 *M* Tris-HCl, pH 8.0, to block the unreacted sites and continue the incubation overnight at 5ºC with rocking.
5. Centrifuge 2 min at 2000*g* in a centrifuge. Separate the supernatant and pellet and save both.
6. Transfer the beads to 15-mL conical centrifuge tubes. Wash two times, centrifuging intermittently with 15 mL of 0.5 *M* NaCl, 50 m*M* MOPS, pH 7.5, followed by 1X wash with 15 mL of 0.1 *M* NaCl, 50 m*M* MOPS, pH 7.5, and suspension in 1.0 mL of the same buffer. Add sodium azide as a preservative at a concentration of 1 m*M* and store the peptide bound beads at 5ºC.
7. To confirm the binding of the peptides to the beads, measure the remaining peptide in the supernatant (*see* **step 5**). Adjust the supernatant to pH 6.0 by addition of 1 *M* HCl and testing with pH paper. Measure the absorbance at 280 nm or other appropriate wavelength and compare with the absorbance of the peptide solution prior to linking to the beads. Efficiency of linkage is usually 80–90%.

For testing of the association of an isolated protein with the peptides linked to beads, the procedure is as follows.

1. The pure protein (10 μg) is added to 1 mL of association buffer, along with 15 μL bead volume of the peptide-linked beads on ice (*see* **Note 7**). Incubation should be for 1.5 h at 5ºC. Beads are then washed 3X in the association buffer with intermittent centrifugation and suspension in fresh association buffer. SDS sample buffer is then added to the precipitates (15 μL of 3X concentrated SDS sample buffer).
2. Samples are heated at 100ºC for 2 min prior to SDS-PAGE. Samples are run on SDS-PAGE followed by Western immunoblotting for the expected associating protein.

For testing the association of a protein in crude lysate with the peptides linked to beads, the following procedure is followed:

1. A 15-μL bead volume of peptide-linked beads is added to 1 mL of a 1 mg/mL cellular extract prepared as in the GST pull-down assay in association buffer (note that association buffer and GST lysis buffer are the same). Incubation is for 1.5 h at 5ºC with rocking. After this time, beads are centrifuged and suspended in fresh association buffer (repeated 3X). SDS sample buffer is then added to the precipitates (15 μL of 3X concentrated SDS sample buffer).
2. Samples are heated at 100ºC for 2 min prior to SDS-PAGE. Samples are run on SDS-PAGE followed by western immunoblotting for the expected associating protein.

3.4. Chemical Crosslinking of Associated Proteins

One method of determining the oligomerization of particular purified proteins is to perform a chemical crosslinking reaction and determine the change in molecular weight, as observed on SDS-PAGE. A compound frequently used for

crosslinking studies is dithiobis[succinimidyl propionate] (DSP). This compound is a water soluble homobifunctional N-hydroxysuccinimide ester that reacts with amino groups of proteins. The linkage can either be left intact, or reduced at the disulfide position to reverse the crosslinking of the proteins. For example, it was shown in our laboratory ***(5)*** that the actin-binding protein Mayven forms a homodimer via BTB/POZ domain association. A generalized procedure for the crosslinking is as follows:

1. Prepare a 0.1–1.0 mg/mL concentration of the protein to be crosslinked in Buffer A.
2. Add DSP to a final concentration of 0.25–5 m*M* such that there is approx 50-fold molar excess of crosslinker in relation to the protein concentration (*see* **Note 8**). The total volume of the reaction mixture should be approx 50 µL.
3. Incubate the reaction for 30 min at 20ºC or for 2 h at 5ºC with rocking.
4. Stop the reaction by the addition of approx 20-fold molar excess of stopping buffer. Continue incubation for 30 min at 5ºC.
5. Add half volume of nonreducing or reducing SDS sample buffer. Heat samples 2 min at 100ºC.
6. Apply to SDS-PAGE the appropriate acrylamide concentration for the molecular weights expected, including appropriate molecular weight standards. Stain with Coomassie blue and destain as usual ***(2)***.

3.5. Photoaffinity Labeling of Associated Proteins

In order to determine the particular binding partner of a protein, for instance, the plasma membrane receptor of a particular extracellular ligand, the technique of photoaffinity labeling can be used. A particular compound that has been used for this purpose is sulfosuccinimidyl 2-(p-azidosalicylamido)ethyl-1-3'-dithiopropionate (SASD). This was used for the determination of binding of carcinoembryonic antigen to macrophages ***(6)***. SASD is a heterobifunctional thiol-cleavable and photo-reactive crosslinker that is radiolabeled at the phenol group. After coupling this labeled compound to the ligand protein, the labeled ligand is photo crosslinked to receptors on the surface of cells. Reduction of the disulfide group of the cross-linker leaves the radioactive tag on the ligand-associated protein. A generalized protocol is as follows.

1. SASD is first dissolved at 0.5 m*M* in PBS, pH 7.4 (*see* **Note 9**).
2. 200 µL of 0.5 m*M* SASD is added to an IODO-GEN precoated tube. 50 µCi of carrier-free ^{125}I sodium iodide in 10 µL of PBS is then added. The reaction is allowed to proceed for 30 s at room temperature in an appropriate iodination hood.
3. The iodinated SASD is then transferred into a tube containing 1 mg of the particular ligand protein in PBS, pH 7.4. Incubation is for 1 h at room temperature. After this time, the sample is applied to a prespun Biospin P6 column (Bio-Rad) (*see* **Note 10**). This removes both the excess Iodine-125 and the unreacted SASD. Quantification of labeling of the ligand is confirmed by counting a small sample (2–5 µL) in a gamma counter.

4. For binding to cell surface receptors, cells are grown to confluence on six-well plates and washed in PBS, or approx 1×10^6 cells are suspended in 1 mL of PBS. To each of these is added 10^6 CPM of labeled ligand. After incubation for 5 min at room temperature, the cells are flashed 5X with a standard photo flash unit.
5. Cells are washed briefly with PBS to remove unbound ligand, and are then scraped onto approx 0.2 mL of 1X reducing SDS sample buffer. Samples are passed 2X through an insulin syringe to disperse DNA, and are then heated for 5 min at 100ºC to break the disulfide bond and leave the ^{125}I label on the target molecule(s).
6. Samples are run on SDS-PAGE *(2)* followed by staining with Coomassie blue, destaining, and drying. The dried gel is exposed to autoradiography film to determine the binding partner of the ligand. As a control, the ligand itself should be run in a separate lane in case there is some high molecular weight component in the ligand that could be confused with the receptor. Also, it is recommended to use a control where excess unlabeled ligand is included to confirm the specificity of binding.

3.6. Plasma Membrane Receptor-Ligand Binding Determination

In order to determine the affinity of a known ligand for a known or unknown plasma membrane receptor, quantitative receptor binding of a radio-labeled ligand can be carried out. The authors have used this procedure to determine the VEGF binding affinity and amount bound to receptors in T47D and MDA-MB-231 breast cancer cells *(7)*.

The ligand protein is specifically labeled with iodine-125 by the following procedure *(8)* (see **ref. *9***):

1. Rinse the polystyrene IODO-GEN pre-coated tube with Tris-Iodination Buffer and drain.
2. Add 2–10 µg of ligand protein in 100 µL of PBS to the Iodogen tube.
3. In an appropriate iodination hood, add approx 1 mCi of carrier-free ^{125}I sodium iodide to the tube containing the ligand protein. Allow the reaction to proceed at room temperature with swirling. Remove the solution from the Iodogen tube, and transfer to a small polypropylene tube.
4. Add 50 µL of scavenger buffer and 150 µL of Tris/BSA buffer.
5. Remove free ^{125}I by applying sample to two Biospin P-6 columns equilibrated with Tris/BSA buffer and spun according to the manufacturer's specifications (apply 150 µL sample per column) (*see* **Note 10**).
6. Sample a small amount of the iodinated protein and run on a 15% acrylamide SDS-PAGE. Expose the stained and dried gel to autoradiography film. Cut out the appropriate band and count using a gamma counter to quantify the ^{125}I incorporation.

Binding of ^{125}I ligand protein to plasma membrane receptors.

1. Grow up cells to confluence in 24-well plates.
2. The ^{125}I ligand is first diluted with unlabeled ligand to a total concentration of 1 m*M*. This diluted ligand should be at a specific radioactivity of 10,000 cpm/pmol.

3. Make up serial dilutions to final concentrations of 0.1 and 10 n*M* of diluted, labeled ligand in binding buffer.
4. Wash cells in 1 mL of binding buffer, drain, and then quickly add 0.5 mL of the serial dilutions of ^{125}I ligand in triplicate.
5. Incubate the cells at 5ºC for 2 h with rocking. Wash the wells 2X with 1 mL of ice-cold binding buffer (*see* **Note 3**). Solubilize the bound ^{125}I ligand by adding 0.25 mL of lysis buffer. Transfer the lysate into polystyrene gamma counting tubes. Also count 0.5 mL of the total ^{125}I ligand to calculate the unbound fraction. Count all tubes in a gamma counter.
6. Plot the bound/free on the ordinate vs. the bound ligand on the abscissa. Calculate the Kd and number of receptors per cell using the Ligand program ***(10)***.

4. Notes

1. Sodium orthovanadate stock solution should be boiled prior to addition to the lysis buffer and lysis buffers should be prepared freshly, not frozen, to ensure the effectiveness of the phosphatase inhibitor.
2. The optimal amount of antibody will vary with the affinity. Often, manufacturers of commercial antibodies have recommendations. Also, it is important to determine from the manufacturer's specifications whether the antibody is able to precipitate the specific protein under such conditions as are to be used.
3. Removal of nonspecific background may require the use of more stringent agents such as 0.5 *M* NaCl or 0.5 *M* LiCl.
4. For phospho-specific antibodies, BSA blocking buffer should be used. For other antibodies, consult the manufacturer's specifications. Other blocking buffers are indicated in Harlow and Lane ***(1)***.
5. It is important never to let the transfers dry when transferring from one solution to another. Thus, moving the transfers as quickly as possible is recommended.
6. Note the difference of the GST lysis buffer compared to immunoprecipitation lysis buffer.
7. Controls should include peptides not expected to associate with the protein and Sepharose beads not linked to peptides. If a phosphopeptide is required for association, a good control is the nonphosphorylated peptide.
8. The volume of DSP should not be greater than 10% of the total volume in the reaction.
9. All steps should be carried out in reduced light conditions.
10. If the molecular weight of the ligand is less than 6 kDa, then the use of other gel-filtration media such as Bio-Gel P-2 or P-4 (Bio-Rad) in a spin column is recommended.

References

1. Harlow, E. and Lane, D. (1988) *Antibodies, A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
2. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.

3. Jhun, B. H., Rivnay, B., Price, D., and Avraham, H. (1995) The MATK tyrosine kinase interacts in a specific and SH2-dependent manner with c-Kit. *J. Biol. Chem.* **270,** 9661–9666.
4. Price, D. J., Rivnay, B., Fu, Y., Jiang, S., Avraham, S., and Avraham, H. (1997) Direct association of Csk homologous kinase (CHK) with the diphosphorylated site Tyr568/570 of the activated c-KIT in megakaryocytes. *J. Biol. Chem.* **272,** 5915–5920.
5. Soltysik-Espanola, M., Rogers, R. A., Jiang, S., Kim, T. A., Gaedigk, R., White, R. A., et al. (1999) Characterization of Mayven, a novel actin-binding protein predominantly expressed in brain. *Mol. Biol. Cell.* **10,** 2361–2375.
6. Toth, C. A. (1992) Carcinoembryonic antigen binding proteins on elicited peritoneal macrophages. *J. Leukoc. Biol.* **51,** 466–471.
7. Price, D. J., Miralem, T., Jiang, S., Steinberg, R., and Avraham, H. (2001) Role of vascular endothelial growth factor in the stimulation of cellular invasion and signaling of breast cancer cells. *Cell Growth Differ.* **12,** 129–135.
8. Riske, F., Hakimi, J., Mallamaci, M., Griffin, M., Pilson, B., Tobkes, N., et al. (1991) High affinity human IgE receptor (Fc epsilon RI). Analysis of functional domains of the alpha-subunit with monoclonal antibodies. *J. Biol. Chem.* **266,** 11,245–11,251.
9. Wood, W. G., Wachter, C., and Scriba, P. C. (1981) Experiences using chloramine-T and 1, 3, 4, 6-tetrachloro-3 alpha, 6 alpha-diphenylglycoluril (Iodogen) for radioiodination of materials for radioimmunoassay. *J. Clin. Chem. Clin. Biochem.* **19,** 1051–1056.
10. Munson, P. J. and Rodbard, D. (1980) Ligand: a versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.* **107,** 220–239.

19

Production of Ligand-Specific Mutants Using a Yeast Two-Hybrid Mating Assay

André Nantel

Abstract

Acquiring functional knowledge from protein–protein interaction studies often necessitates the production of binding-impaired mutants and the study of their effects in biological systems. In many cases, multiple ligands compete for binding to the same protein domain and it becomes useful to produce specific mutations that prevent binding to one ligand but not to the others. We have combined PCR mutagenesis and a two hybrid mating assay to produce a screening strategy that has already proven useful in isolating ligand-specific mutants of the Grb10 and Raf-1 signaling proteins.

Key Words: Protein–protein interactions; mutagenesis; screening; two-hybrid; interaction trap.

1. Introduction

The ability to interact with specific ligands is a significant part of the function of many proteins. Screening for binding partners is often the first step that follows the identification of novel genes and some of the most useful results to arise from the field of functional genomics is the publication of extensive protein–protein interaction maps ***(1–3)***. Proteins commonly have the ability to interact with several ligands and these often compete for the same binding sites. Defining the functional consequences of each of these interactions is a significant challenge. The production of ligand-specific mutants, namely proteins (or protein domains) that are unable to interact with one ligand but bind normally

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to others, is a useful tool to define the functional consequences of these interactions.

One of the most powerful methods for the identification of novel interactors is the yeast two-hybrid/interaction-trap assay ***(4–7)***. As part of our studies of the mammalian adapter protein Grb10, we have combined this method with polymerase chain reaction (PCR) mutagenesis in order to isolate ligand-specific point mutations ***(8)***. A library of mutagenized target proteins is expressed in haploid yeast cells as a fusion to the B42 acidic domain which acts as a transcriptional activator. These cells are mated to another haploid yeast strain which express one of several ligands fused to the yeast GAL4 or *Escherichia coli* LexA DNA-binding domain. In the resulting diploid zygotes, binding between the bait and ligand reconstitutes a functional transcriptional activator that induces the expression of reporter genes such a *LacZ* or *LEU2*. Complete loss of function mutants are easily identified by their inability to interact will all ligands whereas ligand-specific mutants are characterized by an absence of interaction in one strain but not in the others.

We have successfully used this method to isolate mutations in the SH2 domain of the adapter protein Grb10 that fail to interact with specific ligands such as the MEK1 kinase or the insulin receptor tyrosine kinase ***(8)***.

2. Materials

1. Plasmids and strains necessary for the interaction trap assay (OriGene)
   a. Yeast strains EGY48 and RFY20b.
   b. Plasmids pEG202 (*Lex*A fusion), pJG4-5 (B42 Acidic Domain fusion), pSH18-34 (*Lac*Z reporter).
2. PCR Mutagenesis Buffer: 10 m*M* Tris-HCl pH 8.7, 50 m*M* KCl, 100 µg/mL BSA, 0.5 m*M* $MnCl_2$, 4.2 m*M* $MgCl_2$.
3. Taq DNA Polymerase (Amersham Pharmacia).
4. Individual 100 m*M* stocks of dATP, dCTP, dGTP, and dTTP (Amersham Pharmacia).
5. Oligonucleotide primers for amplification and sequencing of bait inserts.
6. Restriction and DNA modification enzymes for subcloning.
7. Agarose gel equipment.
8. Thermal Cycler for PCR amplification.
9. Competent *E. coli* cells.
10. Reagents for yeast transformation or electroporation.
11. 2YT + 100 µg/mL ampicillin plates
12. S Buffer: 10 m*M* KPO_4 pH 7.2, 10 m*M* ethylenediamine tetraacetic acid (EDTA), 50 m*M* 2-mercaptoethanol, 50 µg/mL zymolase 100T (ICN).
13. Lysing Solution: 0.25 *M* Tris-HCl, pH 7.5, 25 m*M* EDTA, 2.5% sodium dodecyl sulfate (SDS).
14. 3 *M* potassium acetate.
15. Facilities for DNA sequencing and analysis.

16. Facilities for the transformation, growth and maintenance of yeast and *E. coli* cultures in liquid and solid media.
17. YPD Media plates
18. The following Complete Minimal (CM) medium dropout plates:
    a. 2% Glucose -*Trp*
    b. 2% Glucose -*His*, -*Ura*
    c. 2% Glucose -*His*, -*Trp*, -*Ura*
    d. 2% Galactose, 1% Raffinose -*His*, -*Trp*, -*Ura*
    e. 2% Galactose, 1% Raffinose -*His*, -*Leu*, -*Trp*, -*Ura*
19. Replica-plating tool and sterile velvets (VWR Canlab) for replicating yeast culture plates.
20. 90-mm round nylon filters.
21. Whatman 3MM paper.
22. Z-Buffer: Add 16.1 g $Na_2HPO_4 \cdot 7H_2O$ (8.5 g anhydrous), 5.5 g $NaH_2PO_4 \cdot H_2O$, 0.75 g KCl, and 0. 246 g $MgSO_4 \cdot 7H_2O$ to 1 L of water. Sterilize by autoclaving.
23. 5-bromo-4chloro-3-indolyl-β-D-galactosidase (Xgal).

3. Methods

The following protocols describe: 1) the production of a mutagenized library for the bait protein; 2) screening of the library against two, or more, ligands; and 3) validation of the binding specificity of the mutants. These methods assume that the researchers are already experienced with handling and transforming yeast cells, and have used the yeast two-hybrid/interaction trap assay to demonstrate specific interactions between the bait and two or more of its ligands. Extensive protocols and notes on the interaction trap (the system currently being used in our lab) are already available in the literature ***(6)*** and the world wide web (http://www.fccc.edu/research/labs/golemis/InteractionTrapInWork.html). In the following example, the Grb10 SH2 domain was mutagenized, fused to the B42 Acidic Domain and screened against *Lex*A-fusions of the carboxy-terminal domains of the Insulin Receptor, the regulatory domain of the Raf-1 kinase or the full length MEK1 kinase (*see* **Note 1**).

3.1. Production of the Mutagenized Library

1. Random PCR mutagenesis of the Grb10 SH2 domain was performed essentially according to Fromant et al. (1995) ***(9)***. This amplification protocol takes advantage of the lack of exonuclease activity in *Taq* DNA Polymerase which prevent correction of random mutations that result from the addition of $MnCl_2$ and an excess of one of the four deoxynucleotide.
    a. Grb10 SH2 domain plasmid 600 p*M*
    b. PCR Mutagenesis Buffer
    c. 5' and 3' PCR primers 0.5 μ*M* each
    d. Forcing dNTP 1.5, 2.0, 2.5, 3.0, or 3.5 m*M*

e. Three remaining dNTPs 0.2 m*M* each
f. *Taq* Polymerase 1 Unit
g. Amplification conditions were 5 min at 94ºC and 16 cycles of 1 min at 91ºC, 30 s at 51ºC, 1 min at 72ºC.

2. In this example, the Grb10 SH2 domain was cloned between the *Eco*RI and *Xho*I sites of pJG4-5. The 1.2 kb insert was amplified by the BCO1 (5' CCA GCC TCT TGC TGA GTG GAG ATG 3') and BCO2 (5' GAC AAG CCG ACA ACC TTG ATT GGA G 3') primers. Each amplification products is flanked by *Eco*RI and *Xho*I sites that were originally present in the plasmid multiple cloning site.
3. Each PCR was verified by agarose gel electrophoresis. If these have produced a DNA fragment of the expected size, purify and pool the amplified products that were obtained with same concentration of forcing dNTP (*see* **Note 2**).
4. If possible, select a pool of mutagenized PCR products in which the highest concentration of forcing dNTP resulted in successful amplifications with all four forcing dNTPs. Cleave with *Eco*RI and *Xho*I, ligate back in the same sites of dephosphorylated pJG4-5, transform in *E. coli* and plate on 150 mm 2YT + 100 µg/mL ampicilin plates. The goal is to obtain approx 10^4–10^5 well-separated colonies.
5. Isolate and sequence sample plasmid inserts from 10 individual colonies to estimate the rate of mutagenesis. It is much better to have a lower rate of mutagenesis than a high number of mutants with more than one mutation per insert. Should the rate of mutagenesis be too high (>1.5 mutations per inserts on average), repeat the subcloning using a pool of mutagenized PCR products produced from a lower concentration of forced dNTP. Once a satisfactory mutagenesis rate has been obtained, the remaining colonies are pooled in 2 mL of 2YT media by scraping with a sterile glass rod, inoculated in 100 mL of 2YT + 100 µg/mL ampicilin and grown for 6–18 h for large scale plasmid purification of the mutagenized library. Once a satisfactory mutagenesis rate has been obtained, this library will be screened against the various ligands.

3.2. Screening Against Ligands

The screening procedure itself is illustrated in **Fig. 1**.

1. Transform *Saccharomyces cerevisiae* strain RFY20b with the Grb10 mutagenized library. Select transformants on 150-mm plates of 2% Glucose -Trp CM media.
2. Patch 1000–5000 individual yeast colonies on 100-mm Glucose -Trp plates (50–100 patches per plate) and grow for 1–2 d at 30ºC (*see* **Note 3**). As a negative control, each plates should also contain at least one patch of RFY20b transformed with the empty pJG4-5 vector.
3. Using sterile velvets, replicate each plates to generate four identical copies (number of copies = number of ligands plus one) which are grown at 30ºC for 1–2 d. One of these plate is kept as a master to allow for the recuperation of interesting clones (*see* **Subheading 3.3.**). The master plates should be wrapped in parafilm and kept at 4ºC.

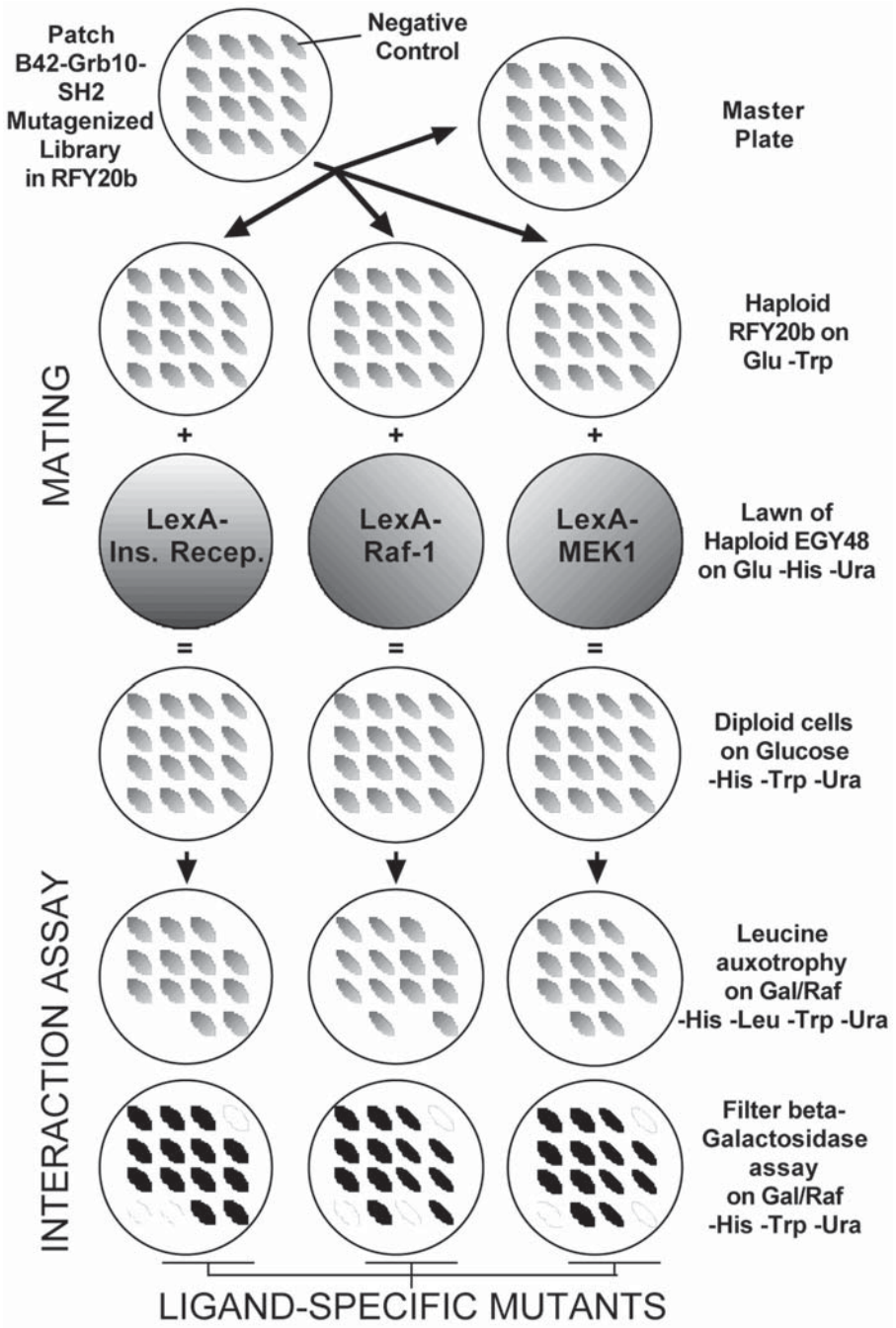

Fig. 1. Overview of the screening strategy for the isolation of ligand-specific mutants by a two-hybrid mating assay.

4. At the same time, prepare plates containing lawns of cells that express the individual ligands fused to the LexA DNA binding domain. We use *Saccharomyces cerevisiae* EGY48 cells previously transformed with the pSH18-34 *Lac*Z reporter plasmid and the ligand plasmids pAN138 (*Lex*A-IR C-terminus), pAN168 (*Lex*A-Raf-1 N-terminus) or pAN104 (*Lex*A-MEK1) ***(8)***. Spread 100 µL of overnight precultures (in 2% Glucose *-His -Ura* CM liquid media) on 100-mm plates of 2% Glucose *-Ura -His* CM. The goal is to obtain a uniform lawn of cells which usually takes 1–2 d of growth at 30ºC.
5. Use replication velvets and YPD plates, mate each of the patches of mutagenized baits with each of the lawns of cells expressing the ligands (*see* **Note 4**).
6. Incubate for 24 h at 30ºC to obtain a light coating of cells. Replica-plate to Glucose *-His*, *-Trp*, *-Ura* CM plates. After 2–3 d of growth at 30ºC, you should see patches of diploid cells which contain all three plasmids.
7. Finally, replica-plate the cells to 2% Galactose, 1% Raffinose *-His*, *-Trp*, *-Ura* CM plates for the beta-galactosidase assays and 2% Galactose, 1% Raffinose *-His*, *-Leu*, *-Trp*, *-Ura* CM plates to test for leucine auxotrophy (*see* **Notes 5** and **6**).
8. Identify patches that fail to grow in the absence of Leucine with one of the ligand but not the others.
9. To confirm results from the growth in the absence of leucine, perform filter beta galactosidase assay on the patches growing on the 2% Galactose, 1% Raffinose *-His*, *-Trp*, *-Ura* CM plates.

3.3. Filter β-Galactosidase Assays

We use the same protocol as Golemis et al. (1999) ***(6)*** (*see* **Note 7**)

1. Place a dry nylon membrane over the yeast patches and wait for it to completely completely wet.
2. Carefully remove the filter and place colony-side up on a Whatman 3MM filter. Partially lyse the cells with 1–3 10-min incubations in a –70ºC freezer (*see* **Note 8**). Place the nylon filter on Whatman 3MM papers saturated with Z Buffer containing 1 mg/mL Xgal.
3. Incubate in 30ºC incubator and monitor for color change.

3.4. Validation and Identification of the Mutations

Plasmids from potentially interesting mutants are reisolated from the patches of RFY20b cells on master plates (prepared in **Subheading 3.1.**) using a protocol obtained from ***(6)***.

1. Briefly spin down cells from a 1-mL overnight liquid culture.
2. Resuspend pellet in 0.5 mL S Buffer.
3. Incubate at 37ºC for 30 min.
4. Add 0.1 mL Lysing Solution and vortex.
5. Incubate at 65ºC for 30 min.
6. Add 166 µL of 3 *M* potassium acetate, vortex, and incubate on ice for 10 min.

7. Spin down in a microcentrifuge at maximum speed for 10 min.
8. Transfer supernatant to a fresh eppendorf tube and precipitate DNA by adding 0.8 mL of cold 95% ethanol.
9. Incubate 10 on ice, spin down in a microcentrifuge for 10 min, and remove supernatant.
10. Wash pellet with 0.5 mL 70% ethanol and dry pellet.
11. Resuspend pellet in 40 µL sterile H_2O.
12. 1–5 µL of this crude miniprep can be used to transform *E. coli* or as a PCR template.
13. Repurify plasmids from a single *E. coli* colony.

It is technically possible to directly sequence the mutated inserts following PCR amplification of the crude extract obtained in **step 11**. We usually prefer to confirm the interactions by retransforming plasmids of each potential mutants in haploid EGY48 cells along with the pSH18-34 *Lac*Z reporter and the *Lex*A-fusion plasmids (*see* **Note 9**). We also quantify binding specificity using commercial liquid β-galactosidase assays.

4. Notes

1. Although the mutagenized library was fused with the Acidic Domain and screened against DNA-binding domains fusions, the reverse is also possible. We have recently used this protocol to screen a library of mutagenized Raf-1 N-terminal domains fused to the *Lex*A DNA-binding domain against acidic domain fusions to either the Grb10 SH2 domain or the activated GTPase RasV12.
2. Purification of PCR products can be done by Ethanol precipitation of with commercially available PCR purification kits.
3. Although we normally produce patches using sterile toothpicks and a printed grid, it has not escaped our attention that this labor-intensive step should be easily amenable to robotization and the use of 96-384 well plates.
4. Mating can be done directly on selective media, but we have observed that the quality of the diploid patches is greatly superior when the cells are given some time to mate and grow under nonselective conditions.
5. If the cell density on the patches is too high, we often see a small amount of growth in the absence of interaction. To prevent this occurrence, we immediately repeat the replica plating by inoculating a new sterile velvet with a plate that had just been itself inoculated.
6. We normally add 1% Raffinose to our 2% Galactose plates as the former is a more easily imported carbon source. We also recommend inoculating the *-His*, *-Trp*, *-Ura* CM plates first to remove excess cells.
7. http://www.fccc.edu/research/labs/golemis/betagal/beta_gal_yeast.html is an excellent source of information on the use of β-galactosidase assays in yeast.
8. Alternatively, float the filter on liquid nitrogen for 5–10 s. Be very careful as they become brittle.

9. This control is necessary to confirm that the new phenotype in "plasmid-dependent". The most common explanation for false positive is the fact that the strength of two hybrid Interactions in diploid cells is often weaker than what is observed in haploids. They are thus more sensitive to the effect of weak mutations.

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References

1. Legrain, P. and Selig, L. (2000) Genome-wide protein interaction maps using two-hybrid systems. *FEBS Lett.* **480,** 32–36.
2. Uetz, P. and Hughes, R. E. (2000) Systematic and large-scale two-hybrid screens. *Curr. Opin. Microbiol.* **3,** 303–308.
3. Ito, T., Chiba, T., Ozawa, R., Yoshida, M., Hattori, M., and Sakaki, Y. (2001) A comprehensive two-hybrid analysis to explore the yeast protein interactome. *Proc. Natl. Acad. Sci. USA* **98,** 4569–4574.
4. Fields, S. and Song, O. (1989) A novel genetic system to detect protein-protein interactions. *Nature* **340,** 245–246.
5. Fields, S. and Sternglanz, R. (1994) The two-hybrid system: An assay for protein-protein interactions. *Trends Genet.* **10,** 195–201.
6. Golemis, E. A., Serebriiskii, I. Finley, Jr., R. L., Kolonin, M. G., Gyuris, J., and Brent, R. (1999) Interaction trap/two-hybrid system to identify interacting proteins, in *Current Protocols in Molecular Biology* (Ausubel, F. M., et al., ed.), Current Protocols, Brooklyn, NY, pp. 20.1.1–20.1.40.
7. Gyuris, J., Golemis, E., Chertkov, H., and Brent, R. (1993) Cdi1, a human G1 and S phase protein phosphatase that associates with cdk2. *Cell* **75,** 791–803.
8. Nantel, A., Mohammad-Ali, K., Sherk, J., Posner, B. I., and Thomas, D. Y. (1998) Interaction of the Grb10 adapter protein with the Raf1 and MEK1 kinases. *J. Biol. Chem.* **273,** 10,475–10,484.
9. Fromant, M., Blanquet, S., and Plateau, P. (1995) Direct random mutagenesis of gene-sized DNA fragments using polymerase chain reaction. *Anal. Biochem.* **224,** 347–353.

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Coimmunoprecipitation Assay for the Detection of Kinase–Substrate Interactions

Lucio Comai

Abstract

Coimmunoprecipitation is a powerful tool to study protein–protein interactions and can be used to test for the physical association between a known protein kinase and its substrate. In this chapter, the author describes a protocol for the preparation of a cell lysate, the immunoprecipitation of the antigen, and the analysis of the immune complex by gel electrophoresis and immunoblotting with antibodies that recognize the putative associated protein.

Key Words: Immunoprecipitation; antibody; protein A/G; immunoblotting; protein interactions.

1. Introduction

Protein coimmunoprecipitation is a method that utilizes antibody-mediated precipitation of antigens to analyze antigen-associated proteins, and it is a powerful assay to test for physical interactions between proteins of interest ***(1)***. This assay can be applied for the analysis of the interaction between a known protein kinase and its substrate, and it is used in combination with other methods to prove a biological interaction. Coimmunoprecipitation assays can be used to test an interaction between two native proteins and/or between recombinant tagged proteins expressed in cells by transfection or infection with recombinant viruses. In the first instance, the precipitating antibody usually recognizes the native proteins, in the latter case, antibodies that recognize the short epitope tags are generally used. The overall strategy involves the capture of an antigen and the associated protein by a specific antibody followed by the precipitation of the

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immune complex by the addition of Protein-A or -G, bacterial proteins with high affinity for the F_c region of IgG and IgM, covalently linked to a solid matrix such as agarose or Sepharose. The critical factor in this assay is the release from the cell of the antigen and associated protein(s) under optimal conditions: extraction conditions that are too gentle may not allow the solubilization of the proteins under examination, while extraction procedures that are too harsh may permanently disrupt a molecular interaction. Parameters such as salt concentration, type and concentration of detergents, duration of incubations, and washing conditions need to be empirically optimized for each antigen and antibody in order to efficiently immunoprecipitate the protein of interest and reduce the nonspecific background. This chapter describes a basic coimmunoprecipitation procedure for the identification of interactions between cellular kinases and their substrates, and involves the following steps (**Fig. 1**).

1. Preparation of the cell lysate under conditions that do not disrupt the protein-protein interactions.
2. Binding of the antibody to the antigen.
3. Adsorption of the antibody-antigen complex to the protein A/G-Sepharose.
4. Collection and analysis of the immune complex.

In the instances where the molecular mass of the coimmunoprecipitated protein is similar to that of the immunoglobulin heavy chain (approx 50–55 kDa), it may be necessary to perform the elution of the associated protein from the beads-bound antigen-antibody complex prior to solubilization to avoid interference from the immunoglobulin heavy chain (*see* **Note 1**). Once a physical interaction between a protein kinase and the substrate has been established, a more detailed characterization of the respective binding domains can be carried out by analysis of deletion and point mutant proteins using coimmunoprecipitation and other biochemical assays.

2. Materials

1. Monolayer or suspension cell culture
2. Phosphate-buffered saline (PBS): 1.37 *M* NaCl, 27 m*M* KCl, 43 m*M* Na_2HPO_4, 14 m*M* KH_2PO_4.
3. Antibodies that recognize the native proteins and/or the polypeptide (epitope) tag (for recombinant proteins).
4. Nutator.
5. Equipment for SDS-PAGE and immunoblotting.
6. Glass dounce homogenizer with type "B" type pestle (optional).
7. Protein A/G Sepharose. Store as a 50% slurry in 70% ethanol at 4ºC.
8. Protease inhibitors (P.I.): 5 µg/mL pepstatin A, 5 µg/mL aprotinin, 5 µg/mL leupeptin, 2 m*M* benzamidine, 0.2 m*M* PMSF (or AEBSF). Store at 4ºC or –20ºC, according to manufacturer specifications.

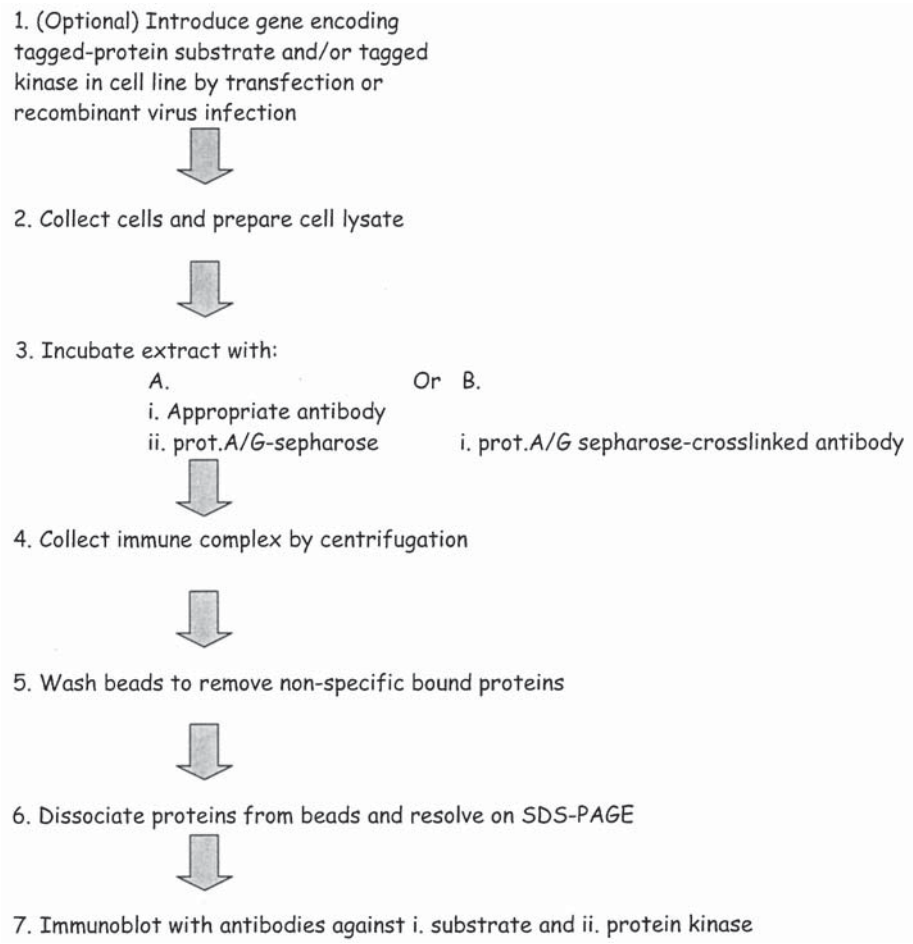

Fig. 1. Flow chart for the coimmunoprecipitation assay.

9. EBC buffer: 20 m*M* Tris-HCl (pH 8.0), 125 m*M* NaCl, 0.5% Igepal, 2 m*M* ethylenediamine tetraacetic acid (EDTA), 20 m*M* NaF, 0.2 m*M* Na_3VO_4. Store at 4ºC.
10. 2X protein sample buffer (2X SB): 62.5 m*M* Tris-HCl, pH 6.8, 10% glycerol, 3% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol. Can be stored at room temperature.

3. Methods

The methods described later outline the preparation of the cell lysate, the coimmunoprecipitation reaction, and the identification of the coimmunoprecipitated partners by SDS-polyacrylamide electrophoresis and immunoblotting (PAGE), in **Subheadings 3.1.–3.3.**, respectively.

3.1. Preparation of the Cell Lysate

1. Place tissue-culture dishes on ice. Remove the culture media and wash the cells twice with cold PBS.
2. Add 5 mL of PBS/100-mm dish and carefully scrape off the cells with disposable cell scraper.
3. Transfer the cells to an ice-cold Eppendorf tube and centrifuge at 750*g* in microcentrifuge for 5 min. Discard supernatant. (If working with suspension cells, collect the cells into a centrifuge tube, centrifuge at 750*g* for 5 min at 4ºC and wash twice with ice-cold PBS before moving to next step).
4. To the pelleted cells add five packed-cell volumes (PCV) of EBC buffer supplemented with protease inhibitors (P.I.) (*see* **Notes 2–3**). Suspend the cells by pipeting up and down gently and incubate on ice for 20 to 30 min with occasional mixing. If working with large volumes, to improve the efficiency of protein extraction transfer the resuspended cells to a glass douncer homogenizer and homogenize with ten up and down strokes using a type "B" pestle.
5. Transfer the protein extract to a cold centrifuge tube and centrifuge at 18,000*g* for 15 min at 4ºC.
6. Transfer supernatant to a fresh tube on ice.
7. Determine the protein concentration of the cell lysate by performing a Bradford assay. Dilute the cell lysate at least 1:10 before determining the protein concentration because of the interference of the detergent in the cell lysis buffer with the Bradford reagent (*see* **Note 4**). Adjust protein concentration to approximately 1 mg/mL.

3.2. Immunoprecipitation

Important: the cell lysates must always be kept cold and all tubes and solutions must be prechilled.

1. Wash the protein A/G sepharose resin twice with ice cold PBS and preequilibrate it in EBC buffer in a 50% slurry (*see* **Notes 5–6**).
2. If the cell lysate was stored at –80º C, thaw the lysate at room temperature and centrifuge at 18,000*g* for 15 min at 4ºC (some proteins may precipitate during the freezing and thawing of the extract) (*see* **Note 7**). Transfer the supernatant (avoiding the pellet) to fresh Eppendorf tube on ice.
3. Preclear the cell lysate by adding 60–80 µL of Protein A/G sepharose slurry (from **step 1**) per 1 mL of lysate and incubate at 4ºC for 10 min on a nutator. Preclearing the lysate will reduce the nonspecific binding of proteins to the resin during the immunoprecipitation.
4. Remove the protein A/G beads by centrifugation at 800*g* at 4ºC for 2 min. Transfer the supernatant to a fresh centrifuge tube.
5. Add the recommended volume of the immunoprecipitating antibody to 300 to 500 µL of cell lysate (approx 300–500 µg of total protein; more lysate may be required for the analysis of less abundant antigens). In general, the amount of antibody used ranges from 1–2 µg, however, the optimal amount of antibody that

will quantitatively immunoprecipitate the protein of interest should be empirically determined for each coimmmunoprecipitation assay (*see* **Notes 8–9**).

6. Incubate the lysate with the antibody for 2 h to overnight at 4ºC on a nutator. A 2 h incubation time is recommended for the immunoprecipitation of most antigens. Longer times may increase the background.
7. Capture the immunocomplex by adding 30 µL of protein A/G Sepharose slurry (15 µL of packed beads) and gentle rocking on a nutator for 2–3 h at 4ºC. In some instances, immunocomplex capture on beads can be enhanced by adding a bridging antibody such as rabbit antimouse IgG. This is especially important with antibodies which bind poorly to Protein A such as antibodies from chicken or goat (*see* **Note 10**).
8. Collect the Sepharose beads by centrifugation (3 min in a microcentrifuge at 800*g*).
9. Carefully aspirate supernatant and avoid beads by leaving approx 20 µL of solution in the tube.
10. Add 800 µL of ice-cold EBC buffer + P.I. to the beads, resuspend by gentle inversion of the tube and incubate on ice for 5–10 min (*see* **Note 11**).
11. Collect the beads by centrifugation (3 min at 800*g*) and aspirate supernatant.
12. Repeat washing **step 10–11**, three more times.
13. After the last wash, centrifuge one more time at 800*g* for 1 min at 4ºC to bring down residual wash buffer that may be on the wall of the tube and carefully aspirate all supernatant.

3.3. Immunoblotting

1. Resuspend the pellet (immunocomplex) in 15–50 µL of 2X sample buffer (2X SB) and mix gently.
2. Heat at 95ºC for 5 min to dissociate the immunocomplex from the beads. Collect the beads by centrifugation and transfer the supernatant fraction to a fresh microcentrifuge tube (supernatant can be stored frozen at –20ºC for later use).
3. Load samples on a SDS-polyacrylamide gel. Load the coimmunoprecipitation reaction in duplicate to allow duplicate blot and detection of each of the two proteins under investigation. Always include in the gel an aliquot of the lysate (input) as a positive control for the immunoblot (*see* **Note 12**).
4. Separate proteins by electrophoresis and blot on nylon or nitrocellulose membrane.
5. Detect coimmunoprecipitated proteins (kinase and substrate) with the appropriate antibodies (*see* **Note 13**).

4. Notes

1. The presence of the immunoglobulin heavy chain in the immunoblot (around 50–55 kDa) can sometimes interfere with the detection of the antigen and/or associated proteins. For this purpose, an additional elution step is recommended for the detection of the associated protein. To elute the coimmunoprecipitated protein from the immune complex, once the immune complex has been washed (**step 13** in the immunoprecipitation protocol; **Subheading 3.2.**), add 50–100 µL of BCO elution buffer [20 m*M* Tris-HCl (pH 8.0), 0.5 m*M* EDTA (pH 8.0), 20% glycerol. Add KCl to 1 *M*,

sodium deoxycolate to 1%, DTT to 1 m*M* and PMSF to 0.2 m*M* just before use] slightly chilled on ice to the beads and incubate on ice for 30 min with occasional gentle flicking of the tube ***(2)***. Centrifuge at 800*g* for 2 min at 4ºC and transfer approx 45 µL of the supernatant to a clean microcentrifuge tube on ice. Add 1/4 volume of ice-cold TCA solution (100% w/v TCA, 4 mg/mL sodium deoxycholate). Vortex tube for 10 s and incubate on ice for 20 min. Centrifuge at 16,000*g* for 10 min at 4ºC. Carefully aspirate supernatant (avoid disturbing the pellet; pellet along the side of the tube may not be visible). Add 100% acetone (approx 700 µL) to wash off the residual TCA, vortex and incubate on ice for an additional 10 min. Centrifuge 18,000*g* for 10 min at 4ºC. Aspirate the supernatant and air-dry the pellet. Resuspend the pellet in 15 µL of 2X protein sample buffer (2X SB). If color turns yellow, add immediately 1 µL of 2 *M* Tris-HCl (pH 8.0). Heat at 95ºC for 3 min prior to loading on a SDS-polyacrylamide gel. Alternatively, the interference from the antibody heavy chain can be avoided by covalently link affinity-purified antibody to the protein A/G–resin prior of the immunoprecipitation; protocols for the affinity purification of antibody and subsequent crosslinking to protein A/G resins can be found in ***(3)***.

2. The composition of cell lysis buffer is a critical variable in the coimmunoprecipitation assay. Ideally, the buffer has to solubilize the kinase and the target substrate and at the same time preserve their association. Whereas buffers such as the radioimmune precipitation assay (RIPA) is the buffer of choice for standard immunoprecipitation assays, it is not suggested for coimmunoprecipitation because it denatures some antigens and, most importantly, it can disrupt protein–protein interactions. In general, the choice of buffer for coimmunoprecipitation depends on the strength of the protein–protein interaction, and requires testing different buffers with varying concentrations of salts and detergents. Buffers containing a concentration of salts between 100 and 150 m*M* and up to 1% of nonionic detergent are the most commonly used and typically yield reproducible results. Nonionic detergents such as Triton X-100 and Igepal CA630 (NP-40) are quite effective in disrupting the cellular membrane and solubilizing the cytoplasm. Increasing salt concentrations are helpful in the extraction of nuclear protein from the nucleus without nuclear membrane disintegration. It is especially important to avoid nuclear lysis, which results in a very viscous solutions, usually leading to high background and nonspecific protein binding. In addition, in any extraction procedure it is critical to minimize proteolytic activity in the cell lysate by keeping the lysate cold (<8ºC) at all times during the procedure and including a cocktail of protease inhibitors to the lysis buffer.
3. To minimize the potential sources of contamination during the immunoprecipitation and/or for enrichment of a specific antigen, it is sometimes recommended to perform the coimmunoprecipitation from a cytoplasmic or nuclear extract rather than whole cell lysate. The following is a conventional protocol for the preparation of nuclear extracts ***(4)***: after washing with PBS, resuspend the cells in four packed cell volumes (PCV) of buffer A [10 m*M* Tris-HCl (pH 7.9), 150 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.5% Igepal CA630] containing 1 m*M* DTT and 0.2 *M* PMSF, and

incubate on ice for 20–30 min (optional: homogenize cells in glass douncer as described in **step 4** of **Subheading 3.1.**). Centrifuge lysate at 1400*g* for 10 min at 4ºC. Collect the supernatant into a fresh tube and save the pellet (nuclei). Add 0.11 volumes of Buffer B [0.3 *M* Tris-HCl (pH 7.9), 1.4 *M* KCl, 0.03 *M* $MgCl_2$] to the supernatant and centrifuge at 100,000*g* for 1 h at 4ºC. Decant supernatant (cytoplasmic S-100 fraction) and dialyze in TM buffer [50 m*M* Tris-HCl (pH 7.9), 12.5 m*M* $MgCl_2$, 1 m*M* EDTA, 10% glycerol] containing 0.1 *M* KCl, 1 m*M* DTT, and 0.2 m*M* PMSF. Pelleted nuclei (from above) are resuspended in 5 PCV buffer C [50 m*M* Tris-HCl (pH 7.5), 0.42 *M* KCl, 5 m*M* $MgCl_2$, 0.1 m*M* EDTA, 10% glycerol, 10% sucrose]. Just before use, add PMSF to 0.2 m*M* and DTT to 1 m*M*. Swirl gently or dounce to resuspend (do not pipette up and down, nuclei may lyse!), and incubate on ice with occasional swirling for 30 min. Centrifuge at 18,000*g* for 30 min at 4ºC, collect the supernatant (nuclear extract) and dialyze in TM buffer (50 m*M* Tris-HCl (pH 7.9), 12.5 m*M* $MgCl_2$, 1 m*M* EDTA, 10% glycerol) containing 0.1 *M* KCl, 1 m*M* DTT and 0.2 m*M* PMSF.

4. The efficacy of cell breakage and protein extraction can affect the yield of specific antigens. Verify both parameters at the onset of an experiment before proceeding with the immunoprecipitation. Extraction conditions for each antigen should be evaluated by immunoblotting of small-scale extract preparations that vary concentrations of salt and nonionic detergent.
5. It is recommended to cut off the pipet tip before handling agarose/sepharose beads because they can plug small bore tips.
6. Protein A-Sepharose is our resin of choice, however it can be substituted with protein A-agarose or protein G-Sepharose. In our experience, coimmunoprecipitations with agarose beads tend to produce a higher background than sepharose. Protein G-Sepharose is generally used for the absorption of goat antibody and for some mouse IgG isotypes.
7. In many instances, particularly when problems with high background are present, it is suggested to preclear the lysate by centrifugation at 100,000*g* for 30 min at 4ºC. Under these conditions, aggregates of denatured proteins, which tend to be a cause of increased background, are efficiently cleared from the lysate.
8. In general, since a protein kinase may interact with more than one substrate, it is suggested to perform the immunoprecipitation with antibodies that recognize the substrate and then detect the associated protein kinase (although it is desired to confirm a protein interaction by immunoprecipitation of both partners).
9. When coimmunoprecipitation assays are used to assess interactions between recombinant proteins, it is useful to generate constructs that express differentially tagged proteins. Short polypeptides that are recognized by high affinity monoclonal antibody are extremely useful for this type of analysis. The epitope tag is usually added to the amino- or carboxy-terminus. Three widely used polypeptide tags are HA, c-*myc*, and Flag ***(5–7)***. Monoclonal antibodies (anti-HA, -*myc*, and –Flag) and affinity resins (anti-Flag-agarose) are commercially available from several suppliers.
10. Preabsorption of antibody to protein A/G beads may be necessary when the required volume of antibody is excessive (such as when working with supernatants from a

hybridoma cell line). In this case, the antibody is first absorbed to the resin by incubation of the hybridoma supernatant with protein G beads. The cell lysate is then added to the antibody-protein G beads that have been preequilibrated in the appropriate buffer.

11. Washing conditions have to be defined empirically. Milder buffers (such as PBS) may yield higher background whereas high stringency conditions (such as RIPA) can dissociate the interacting protein from the immunoprecipitated complex. In general, it is suggested to start from the same buffer used to lyse the cells, and then adjust the conditions (such as salt and detergent concentrations) later, according to the results of the immunoblot.
12. Control immunoprecipitation reactions are essential for confirming or ruling out a specific interaction between a protein kinase and a substrate. To control for specificity we generally use preimmune serum and an antibody against an unrelated protein. It is important that the type and source of the control antibody is the same as the antibody tested (i.e., polyclonal vs polyclonal; monoclonal vs monoclonal; ascites fluid vs ascites fluid; hybridoma supernatant vs hybridoma supernatant; rabbit sera vs rabbit sera, etc.). When tagged proteins are analyzed, proper control may include immunoprecipitations from an extract expressing an untagged version of the protein, immunoprecipitation from an extract expressing an unrelated tagged protein and/or immunoprecipitation with an antibody that recognizes a different epitope tag.
13. As a final consideration it is important to remember that unless the interaction can be demonstrated between purified components, the involvement of a bridging factor cannot be ruled out.

References

1. Phizicky, E. M. and Fields, S. (1995) Protein-protein interactions: methods for detection and analysis. *Microbiol. Rev.* **59,** 94–123.
2. Gu, W., Shi, X. L., and Roeder, R. G. (1997) Synergistic activation of transcription by CBP and p53. *Nature* **387,** 819–823.
3. Harlow, E. and Lane, D. (1999) Using antibodies: a laboratory manual. *Cold Spring Harbor Laboratory* (2nd ed.) Cold Spring Harbor Press.
4. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* **11,** 1475–1489.
5. Evan, G. I., Lewis, G. K., Ramsay, G., and Bishop, J. M. (1985) Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol. Cell Biol.* **5,** 3610–3616.
6. Hopp, T. P., Prikett, K. S., Price, V., Libby, R. T., March, C. J., Cerretti, P., et al. (1988) As hort polypeptide maker sequence useful for recombinant protein identification and purification. *Biotechnology* **6,** 1205–1210.
7. Niman, H. L., Houghten, R. A., Walker, L. E., Reisfeld, R. A., Wilson, I. A., Hogle, J. M., and Lerner, R. A. (1983) Generation of protein-reactive antibodies by short peptides is an event of high frequency: implications for the structural basis of immune recognition. *Proc. Natl. Acad. Sci. USA* **80,** 4949–4953.

IV

Genomic Rearrangements

21

Mutational Analysis of the Androgen Receptor Using Laser Capture Microdissection and Direct Sequencing

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Abstract

Molecular analysis of prostate cancer specimens is complicated by tumor heterogeneity and admixture of malignant cells with benign epithelium, stroma and inflammatory cells. Laser Capture Microdissection allows procurement of selected groups of cells from specific areas of tissue sections. We have used Laser Capture Microdissection to obtain pure samples of malignant prostate epithelial cells from frozen radical prostatectomy specimens. Methods for DNA extraction, androgen receptor amplification and exon sequencing have been optimized. These methods should be adaptable for molecular analysis of DNA, RNA and protein from other complex tissues.

Key Words: Laser capture microdissection; androgen receptor; mutations; prostate cancer; polymerase chain reaction; direct sequencing.

1. Introduction

The development and growth of the prostate depends upon androgens. Prostate cancer is the most common cancer in American men ***(1)***. Prostate cancer responds to medical or surgical castration with clinical remission that may last several years. However, prostate cancer eventually recurs in an androgen-independent form that is rapidly fatal. The androgen receptor may play an important role in the development and progression of prostate cancer ***(2)***. Molecular analysis of the androgen receptor is difficult owing to the need for carefully

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preserved tissue (the prostate is a factory of proteolytic enzymes), crosslinking of DNA by routine fixation and processing, the admixture of benign and malignant tissues, tumor heterogeneity and the size, GC-richness, and conformational complexity of the androgen receptor gene.

The identification of the 877 Thr→Ala mutation in the hormone binding domain of the androgen receptor gene of the androgen-sensitive LNCaP human CaP cell line initiated a search for gene mutations in radical prostatectomy specimens from which androgen-independent prostate cancer might derive ***(3,4)***. Tilley et al. used paraffin-embedded tissues, DNA PCR and single-strand conformational polymorphism (SSCP) to screen for mutations ***(5)***. They were the first group to study the technically demanding A exon where 50% of the mutations were found. Overall, androgen receptor mutations were found in 44% of 25 patients who underwent transurethral resection for urinary retention prior to androgen deprivation therapy. Tilley's findings remain controversial since these results have not been duplicated by Takahashi et al. using the same methodology in prostatic archival prostate cancer tissue ***(6)***. Using the Tilley method, but in frozen tissue and in only exons B-H, two groups found one mutation in 30 patients studied ***(7,8)***. Newmark et al. used frozen tissue, DNA PCR and denaturing gradient gel electrophoresis (DGGE) to screen for mutations in exons B-H ***(9)***. A point mutation was found in the androgen receptor steroid binding domain in 1 of 26 radical prostatectomy specimens examined.

Culig and associates described the first mutant androgen receptor detected in androgen-independent prostate cancer ***(10)***. A point mutation in the steroid binding domain was identified in a prostatic aspirate from one of seven patients studied. Taplin et al. found numerous single base changes when cDNA produced by reverse transcription polymerase chain reaction (RT-PCR) from aspirated bone marrow specimens was cloned and sequenced; overall, androgen receptor mutations were found in 5 of 10 patients studied ***(11)***. Using SSCP of PCR amplified exons B-H only, mutations were found rarely by other investigators ***(7,8, 12,13)***. Gaddipati et al. reported the LNCaP mutation in 6 of 24 patients studied by direct DNA sequencing of PCR amplification of exons 4-8 ***(14)***; however, this proved to be PCR error (personal communication of Dr. Srivastava).

Technical problems because of Taq polymerase errors, differences in the sensitivity of analysis for mutations using SSCP versus DGGE ***(9,15)*** vs direct sequencing (**Fig. 1**), difficulty of analysis of the large and GC-rich A exon (most investigators analyze only exons B-H) and theoretical concerns regarding the validity of examining bone marrow metastases render these findings controversial. However, androgen receptor gene expression is maintained in both androgen-dependent and androgen-independent prostate cancer. Moreover, of the 7 androgen receptor mutations functionally analyzed thus far, all are active transcription factors and exhibit altered ligand-binding specificity that might enhance

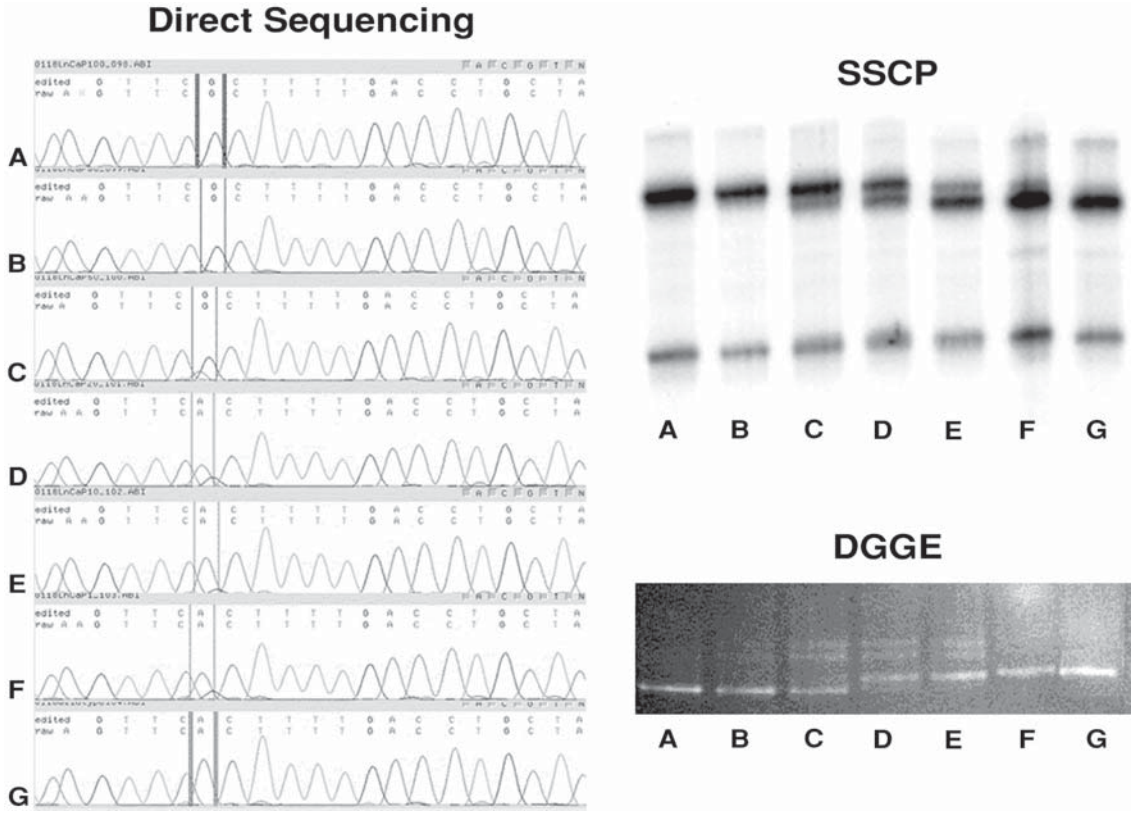

Fig. 1. The sensitivity of direct sequencing, denaturing gradient gel electrophoresis (DGGE) and single-strand conformational polymorphism (SSCP) were compared using a series of mixtures of wild type and LNCaP exon H PCR product. The 877 Thr→Ala mutation can be detected by each of the techniques when mutant PCR product represents at least 20–50% of the total PCR product. Direct sequencing relies upon distinguishing the presence of a nucleotide peak from background. The level of the threshold set for automated labeling of nucleotides may decrease further the sensitivity of direct sequencing (SSCP, DGGE, and direct sequences: **A** = 100% mutant; **B** = 80% mutant; **C** = 50% mutant; **D** = 20% mutant; **E** = 10% mutant; **F** = 1% mutant; and **G** = 100% wild type).

their responsiveness to adrenal androgens, other steroids or anti-androgens *(3,4, 8–12,17,18)*. That these mutations retain androgen-dependent transcriptional activity suggests that functional androgen receptor is required for growth of prostate cancer even in the absence of androgen and that gain of function mutations may serve as a mechanism for progression to androgen-independence in a significant number of men with prostate cancer.

Laser capture microdissection (LCM) is the product of a collaborative effort between scientists at the National Institutes of Health and Arcturus Engineering, Inc. *(19)*. LCM has recently been developed to a point that it is semi-automated, standardized and operator friendly. This new technology has been applied to the study of prostate cancer *(20)*.

LCM uses a thermoplastic film (made of an ethylene vinyl acetate polymer) that is bonded to a plastic cap *(21)*. Once the operator has chosen the cells of interest, a carbon dioxide laser delivers a short duration, focused pulse that heats the thermoplastic film. The heated polymer coating bonds to the targeted cells, which are subsequently transferred from the glass slide to the cap when the cap is lifted from the slide by means of the placement arm at the end of the microdissection. The operator then transfers the cap to the tube containing the extraction buffer from which DNA, RNA or protein can be obtained for further analyses. With the addition of a new cap onto the arm, the operator can capture more cells from the same section or proceed to another section. By adjusting the spot size and the duration of the pulse, the operator can choose to capture from one cell to thousands of cells.

LCM has been instrumental for evaluating androgen-dependent prostate cancer for the presence of androgen receptor mutations that may contribute to the development of androgen-independent prostate cancer. Samples of benign epithelial cells and prostate cancer cells can be microdissected from the same radical prostatectomy specimen of clinically localized prostate cancer. DNA from both samples can be extracted, centrifuged, aliquoted and stored at –20ºC for as long as 6 mo. The subsequent PCR is straightforward except that PCR conditions vary among exons and exon fragments. Microdissected cells from paraffin-embedded tissues have been analyzed using the same methods; however, sequence results have been poor. This chapter describes a step-by-step protocol for LCM, DNA extraction from LCM procured cells and DNA amplification for sequencing of the androgen receptor gene.

2. Materials

2.1. Laser Capture Microdissection

1. PixCell® II Laser Capture Microdissection Instrument with PixCell II Image Archiving Workstation, 110V (Arcturus, Mountain View, CA).
2. Cryostat.

3. Daigger Superfrost®/Plus Microslides (Daigger and Company, Wheeling, IL).
4. CapSure™ LCM Caps (Arcturus).
5. Mayer's Hematoxylin (Sigma, #MHS 16, St. Louis, MO).
6. Eosin Y (CellPoint Scientific, #1B 425, Gaithersburg, MD).
7. Phloxine B (Fisher Scientific, #P-387, Raleigh, NC).
8. Lithium Carbonate (Sigma, #L-4283).
9. Freezer for −80ºC.

2.2. DNA Extraction and PCR for the Human Androgen Receptor Gene

1. An oven or incubator that can be set to 55ºC.
2. Make fresh 2.5-mg/mL of Proteinase K solution. Add 2.5-mg powdered Proteinase K (Sigma, #P2308) to 1.0 mL of RNAse/DNAse free water (Gibco-BRL, Carlsbad, CA) in a sterile 1.5-mL centrifuge tube on ice. Aliquot this mixture into 20-µL portions in 1.5-mL centrifuge tubes, cover each tube with foil, and store in a −20ºC freezer for up to 1 mo.
3. Digestion buffer. Mix thoroughly and store in the 4ºC refrigerator for up to 6 mo. 500 µL of 1.0 *M* Tris-HCl pH 8.0 (Gibco-BRL); 50 µL of 0.5 *M* ethylenediamine tetraacetic acid (EDTA) pH 8.0 (Gibco-BRL); 500 µL of Tween 20; 48.95 mL of RNAse/DNAse free water (Gibco-BRL).
4. Cap insertion tool (Pix Cell-II Cap Insertion Tool, Arcturus, cat. #LCM0501).
5. CR Thermal cycler.
6. Taq Gold™ PCR kit (Applied Biosystems, Branchurg, NJ).
7. Advantage-GC Genomic PCR Kit (Clontech Laboratories, Inc.; Palo Alto, CA; cat #K1908-1,-y).
8. Gel electrophoresis equipment.
9. Primers for PCR (*see* **Tables 1** and **2**).

3. Methods

The methods described below will outline 1. the laser capture microdissection procedure, 2. the extraction of DNA from the microdissected cells, and 3. and 4. the amplification of the androgen receptor DNA for direct sequencing.

3.1. Laser Capture Microdissection

3.1.1. Embedding Frozen Tissue

1. Set the cryostat to −24ºC before starting the procedure.
2. Place an empty cryomold on dry ice for 5 min and then transfer it to the inside of the cryostat.
3. Cover the bottom of the cryomold with Optimum Cutting Temperature (OCT) embedding media (Sakura, cat #4583, Torrance, CA).
4. Place the frozen tissue such that the tissue is flush against the bottom of the cryomold.
5. Fill the cryomold with embedding media and allow it to harden in the cryostat.

Table 1
Primers Used for AR Exons Amplification[1]

PCR region[1]	GeneBank Accession #	Position of primer in exon region (base pair)	Primer name[1]	Sequence of primer 5' → 3'[1]	PCR program
EXON A[1]	M27423				
A1		2-22	A1- 5'	GCCTGTTGAACTCTTCTGAGC	1
		427-406	A1- 3'	GCTGTGAAGGTTGCTGTTCCTC	
A2		384-403	A2- 5'	CACAGGCTACCTGGTCCTGG	2
		799-778	A2- 3'	CTGCCTTACACAACTCCTTGGC	
A3		721-741	A3- 5'	GCTCCCACTTCCTCCAAGGAC	3
		1248-1228	A3- 3'	CGGGTTCTCCAGCTTGATGCG	
EXON B (2)	M27424	1-24	B- 5'	GCCTGCAGGTTAATGCTGAAGACC	3
		379-354	B- 3'	CCTAAGTTATTTGATAGGGCCTTGCC	
EXON C (3)	M27425	101-123	C- 5'	TTATCAGGTCTATCAACTCTTGT	3
		413-391	C- 3'	CTGATGGCCACGTTGCCTATGAA	
EXON D (4)	M27426	97-119	D- 5'	GATAAATTCAAGTCTCTCTTCCT	1
		456-434	D- 3'	GATCCCCCTTATCTCATGCTCCC	
EXON E (5)	M27427	1-25	E- 5'	CAACCCGTCAGTACCCAGACTGACC	3
		285-260	E- 3'	AGCTTCACTGTCACCCCATCACCATC	
EXON F (6)	M27428	1-24	F- 5'	CTCTGGGCTTATTGGTAAACTTCC	2
		294-272	F- 3'	GTCCAGGAGCTGGCTTTTCCCTA	
EXON G (7)	M27429	1-24	G- 5'	CTTTCAGATCGGATCCAGCTATCC	3
		416-393	G- 3'	CTCTATCAGGCTGTTCTCCCTGAT	
EXON H (8)	M27430	1-21	H- 5'	GAGGCCACCTCCTTGTCAACC	4
		347-322	H- 3'	GGAACATGTTCATGACAGACTGTACACTA	

[1]Exon nomenclature and sequences of primers were taken from **ref. 22**.

Table 2
Primers Used for Amplification GC-rich AR Exon A4 Region[1]

PCR region	GeneBank Accession #	Position of primer in exon region (base pair)	Primer name	Sequence of primer 5' → 3'
EXON A[1]	M27423			
A4		1149-1175	A4-5'[2]	CCAGAGTCGCGACTACTACAACTTTCC
		1753-1731	A4-3'[2]	CCAGAACACAGAGTGACTCTGCC
A4		1157-1181	GGN-5	GCGACTACTACAACTTTCCACTGGC
		1680-1655	GGN-6	GTAAGGTCCGGAGTAGCTATCCATCC
A4		1360-1386	GGN-7	CCGCTTCCTCATCCTGGCACACTCTC
		1625-1600	GGN-8	GGACTGGGATAGGGCACTCTGCTCAC
A4		1163-1189	GGN-9	ACTACAACTTTCCACTGGCTCTGGCCG
		1751-1726	GGN-10	AGAACACAGAGTGACTCTGCCCTGGG
A4		1163-1189	GGN-9	ACTACAACTTTCCACTGGCTCTGGCCG
		1698-1673	GGN-12	AACTTACCGCATGTCCCCGTAAGGTC

[1]*See* p. 298 for two-step PCR conditions A4 region.
[2]Sequences of primers were taken from **ref. 22**.

3.1.2. Cutting Sections

1. Remove the frozen block from the cryomold, trim the block to fit the chuck (SLEE GMBH, Mainz, Germany) and attach the block to the chuck by covering it in OCT embedding media (Sakura, cat #4583, Torrance, CA).
2. Allow the block to equilibrate to the temperature of the cryostat before cutting.
3. Use disposable blades to do the sectioning.
4. Cut two 6-µm serial sections of prostate tissue onto the middle of a positively charged glass slide.
5. Leave the glass slides in the cryostat or on dry ice until all 20 slides are ready to be stained. This protocol allows for 20 slides to be used for various reasons explained later.
6. Slides 1–3, 6–8, 11–13, and 16–18 are used for LCM. These slides will be stained but not coverslipped and will be kept in a dessicator.
7. Slides 4, 9, 14, and 19 will be stained with hematoxylin and eosin (H&E) and coverslipped. These slides will later serve as morphology reference slides.
8. Slides 5, 10, 15, and 20 will be fixed for 14 s then transferred to an immunohistochemical technician for high molecular weight cytokeratin staining to assist the pathologist in evaluation of the standard stains.

3.1.3. Staining Sections

1. Fix the slides in 70% ethanol for 1 min.
2. The staining procedure is as follows:
3. Distilled water (15 s).
4. Mayer's Hematoxylin (15 s) that must be filtered before each use.
5. 0.5% Lithium carbonate in distilled water (30 s).
6. Distilled water (30 s).
7. 70% ethanol (30 s).
8. Alcoholic Eosin Y with Phloxine that must be filtered before every use (30 s).
9. 95% ethanol (30 s).
10. 100% ethanol (30 s).
11. 100% ethanol (30 s).
12. Xylene (1 min).
13. Xylene (1 min).
14. Air dry completely (2–4 min).

3.1.4. Microdissection

1. Take the CapSure™ LCM caps from a bench top dessicator and load into the Capsure™ cassette module.
2. For single cell capture, set the laser spot size to 7.5 µm, the laser power to 30–40 mW, and the firing time from 800 µs–2.0 ms.
3. For multiple cell capture, set the laser spot size to 15 µm, the laser power to 32–55 mW and the laser firing time to 900 µs–3.2 ms.

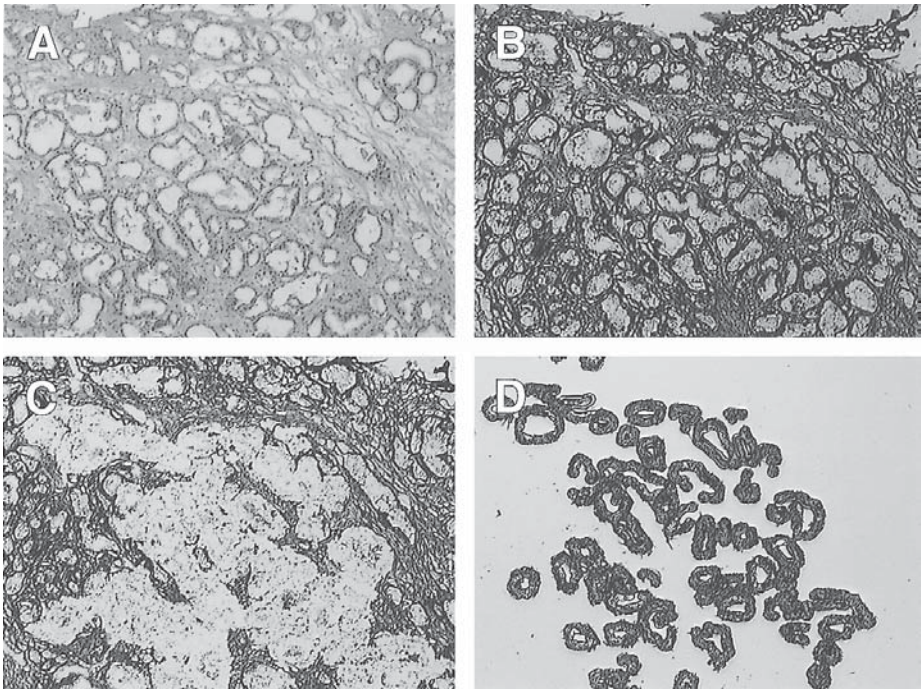

Fig. 2. Photomicrographs illustrating LCM performed on Gleason pattern 3 adenocarcinoma of the prostate. (**A**) Hematoxylin and eosin stained tissue containing malignant glands (slide used as a morphological reference only); (**B**) Mayer's Hematoxylin and eosin stained tissue prior to LCM (notice the laser pointer can be visualized in the center of the photomicrograph); (**C**) The tissue remaining after LCM and thermoplastic cap transfer of malignant glands of interest; (**D**) Malignant glands displayed on the thermoplastic cap after removal by LCM.

4. Place the glass slides with the stained sections on the microscope podium.
5. Using the cap arm, place the cap over the stained section.
6. Visualize the section on the computer monitor and use the laser pendant switch to capture the cells desired.
7. Use one cap to capture malignant cells and another cap to capture benign cells for each sample (**Fig. 2**).
8. Microdissect up to 5000 cells per sample which can fit on one cap.
9. The individual caps are inserted into a sterile 0.5-mL centrifuge tube and placed on dry ice until all sections have been microdissected.
10. Once all the sections have been microdissected, the samples are either immediately processed or kept in the −80ºC freezer for no longer than a week.

3.2. Extraction of DNA from LCM Caps

1. The LCM caps attached to a 500-µL centrifuge tube on dry ice are transferred to an ice bucket.
2. Prepare extraction buffer (the extraction buffer contents and many other helpful technical suggestions were provided by Dr. John Gillespie at the National Cancer Institute) by pipetting 480 µL of digestion buffer into the 1.5-mL centrifuge tube containing the 20 µL Proteinase K, mix by pipetting and leave on ice.
3. Label clean 0.5-mL LCM centrifuge tubes and then add 50 µL of the extraction buffer to each centrifuge tube.
4. Remove the LCM cap from the original centrifuge tube using the Cap Insertion Tool and place the cap on top of the tube containing the extraction buffer.
5. Allow the samples to sit at room temperature for 10 min. Turn the tubes upside down to bring the extraction buffer in full contact with the cap. Wrap a strip of Paraffilm™ (American National Can, Menasha, WI) around the tube and cap to keep the cap from falling away from the tube in the 55º oven or incubator.
6. Place the tube upside down so that the cap is in contact with the bottom of the oven and incubate for 3 h at 55ºC.
7. After 3 h, remove the tubes from the oven, take off the Paraffilm™ and spin the labeled centrifuge tubes for 5 minutes at 2040*g*.
8. Remove the tubes and place on ice as you aliquot 15 µL portions into two 0.5-mL tubes. Store the aliquots in a −20ºC freezer.

3.3. PCR of DNA Derived from LCM

1. Take the Taq Gold™ DNA Polymerase kit out of the −20ºC freezer and transfer all the components of the kit to the labtop cooler (Nalgene, Duluth, GA).
2. Thaw the 10X PCR (the 10X Buffer contains 150 m*M* Tris-HCl pH 8.0 and 500 m*M* KCl) Buffer, 25 m*M* $MgCl_2$, 10 m*M* dNTP mix, 20 µ*M* forward and reverse primer sets in an Eppendorf rack to thaw.
3. Once the PCR components are completely thawed, vortex briefly and centrifuge all components including the Taq Gold before placing components on ice. Keep Taq Gold, which is always left in the −20ºC labtop cooler at all times when not in use, as degradation will occur if allowed to come to room temperature for even short periods of time.
4. Make a "Master Mix" of the PCR components such that final concentration of each component in a 100-µL PCR reaction is as indicated later. Multiply all the components by the number of PCR samples and add one to insure that there is enough Master Mix for all the samples. Add enough micropure water to bring to a total volume of 100 µL in each reaction. 1X PCR Gold Buffer. 1.5 m*M* $MgCl_2$. 0.2 m*M* dNTP mix that contains 2.5 m*M* of dATP, dCTP, dGTP, and dTTP. 0.2 µ*M* solution of reverse and forward primers. 0.05 U of Taq Gold polymerase. Your Master Mix chart might look like this for a 100-µL reaction:
   a. ( ) µL of MicroPure H_2O (water will vary depending on volume of DNA);
   b. 10 µL of 10X PCR Gold Buffer;

Table 3
PCR Conditions for Amplification of AR Exon and Exon Fragments

PCR program	Primer sets	Hot start	PCR cycle			Final extension
			Denature	Annealing	Extension	
1	A1, D	95ºC 10 min	95ºC 1 min	58ºC 90 s	72ºC 90 s	72ºC 10 min
2	A2, F	95ºC 10 min	95ºC 1 min	57ºC 1 min	72ºC 90 s	72ºC 10 min
3	A3, B, C, G, E	95ºC 10 min	94ºC 1 min	60ºC 1 min	72ºC 90 s	72ºC 10 min
4	H	95ºC 10 min	94ºC 90 s	57ºC 90 s	72ºC 150 s	72ºC 10 min

c. 6 µL of 25 m*M* $MgCl_2$;
d. 2 µL of 10 m*M* dNTP mix;
e. 1 µL of 20 m*M* solutions of each forward and reverse primer (*see* **Table 1**);
f. 1 µL of 5 U/µL of Taq Gold.

5. Return all of the components for the PCR to the labtop cooler and then a –20ºC freezer.
6. Pipette enough of the Master Mix into each of the reaction tubes to have a 100-µL total reaction volume after the DNA has been added.
7. Take an aliquot of the LCM extracted DNA from the –20ºC freezer and thaw on ice.
8. When the samples are thawed, take 2–4 µL (200–400 cells) of the DNA and add to the PCR mix for each sample, keep the samples on ice, and return them to the –20ºC freezer immediately.
9. Close the cap on each sample, set the appropriate PCR program (*see* **Table 3**) and place the tubes in a thermal cycler.
10. Taq Gold DNA polymerase requires an 8–10 min at 95ºC "hot" start in the PCR machine to activate the enzyme. Perform amplification according to the programs described in **Table 3** for GeneAmp PCR System 9600 (Perkin Elmer, Wellsley, MA).
11. After the PCR is completed, analyze the amplified samples. Run 15 µL of each PCR product and DNA size markers on a 1.2% agarose gel with ethidium bromide staining. Compare the size of the PCR products to the markers.
12. If PCR was successful, "clean-up" the PCR products to prepare them for the sequencing facility.

3.4. PCR Protocol Specific for the Androgen Receptor A4 Region

Androgen receptor A4 region contains GC rich sequences [$GGN_{6,}$ GGC_n, where **n** is usually in the range of 15–18]. This region has strong propensity to form secondary structure, which leads to difficulties in PCR-amplification and sequencing. Successful amplification of [$GGN_{6,}$ GGC_n] repeats has been performed using the Advantage-GC Genomic PCR Kit (Clontech Laboratories, Inc.). The primers pairs at least 25 nucleotides long was designed to optimize T_m (close to 70ºC) and GC content (50–60%) (*see* **Table 2**). PCR was performed using five different pairs of primers. In each case full size product was reproducibly amplified. Sequencing of each product gave unambiguous results consistent in all overlapping regions between different PCR products.

1. **Two-Step PCR** was performed on GeneAmp PCR System 9600 (Perkin Elmer) using the following conditions:
   - 94ºC for1 min
   - 35 cycles:
     94ºC for 30 s
     68ºC for 3 min
   - 70ºC for 5 min
   - Soak at 15ºC
2. **PCR reaction Mix** for total volume 50 µL:
   - 10.0 µL—DNA template;
   - 14.8 µL—MicroPure H_2O;
   - 10.0 µL—5X GC Genomic PCR Reaction Buffer;
   - 2.2 µL—25 m*M* $Mg(OAc)_2$;
   - 10.0 µL—GC Melt (5 M);
   - 0.5 µL—Forward primer 20 m*M*;
   - 0.5 µL—Reverse primer 20 m*M*;
   - 1.0 µL—50X dNTP mix (10 m*M* of each);
   - 1.0 µL—Advantage-GC Genomic Pol. Mix (50X).

4. Notes

4.1. Laser Capture Mircodissection

1. Slides that carry a positive charge need to be opened 2 wk prior to use. A slide that is not allowed to sit before use will hold the tissue too firmly and prevent the easy lifting of cells onto the cap.
2. New disposable blades must be used to section each block.
3. All the equipment and the area around the cryostat must be wiped down with 100% ethanol.
4. Gloves are worn throughout the procedure to reduce contamination.
5. Care must be taken in the transference of frozen tissue if the LCM equipment is not located within the same laboratory as the tissue storage facility. If there is a need to transport the tissue, even for short distances, an insolated container of dry

ice must be utilized. The tissue must be kept in a frozen state (greater than –20ºC) at all times to prevent enzymatic degradation of the DNA or RNA. These principles must also be practiced in handling the transference of the caps at the completion of LCM.

6. Samples are cut, stained and microdissected the same day for optimal DNA recovery. If the slides are kept at –80ºC for up to three days, the extraction and yield of DNA is adequate; storage longer than three days reduces the yield such that sequencing is often not possible. Once the sample is obtained, storage at –80ºC for longer than 7 d prior to processing (to allow processing many samples at one time in batch fashion) may decrease yields significantly.
7. The morphology of tissue structures is more clearly visible when the sections are approx 6-µm thick. Above 10 µm thickness, the structures are more difficult to discern.
8. Use the lowest acceptable laser power and firing time. The settings required will vary from day to day and from section to section. If the film on the cap does not pick up the selected cells, the laser power was probably too low to warm the film sufficiently. In that case, increase the laser power as high as to 60 W, since settings above 60 W may damage the cells and their DNA.
9. The cells may fail to lift if the microscope slide is wet; complete cell desiccation is necessary for the LCM technique. Slides may be put back into xylene which contains 4 Å molecular sieve granules for 2–4 min and air desiccated to improve cap adherence.

4.2. Extraction of DNA From LCM Caps

1. Because the caps will be on dry ice, it is important to let the cap equilibrate to room temperature before placing it in the presence of the DNA extraction buffer. This step will allow the cap to expand to its natural shape which is important for it to fit properly on the top of the 0.5-mL centrifuge tube containing the extraction buffer.
2. Once the cap is on the 0.5-mL centrifuge tube containing the extraction buffer, wrap Paraffilm™ around the cap and tube together. If the cap becomes detached from the tube, the Parafilm™ will prevent loss of extracted material and buffer. Please take care to properly insert the cap onto the tube. It is highly recommended to use the Cap Insertion Tool described earlier to properly handle the cap. The Cap Insertion Tool also has a calibrated surface that protects against placing the cap too far into the centrifuge tube. Touching the inner surface of the cap, even with latex gloves, may contaminate the contents or result in the loss of cells. Improper cap insertion will also not allow for a sufficient waterproof seal to form and the valuable contents of the tube may be lost. There are commercial 0.5-mL centrifuge tubes available with line makers inside of the tube to indicate when the cap has been inserted too far into the tube to allow for a sufficient waterproof seal.
3. If, after the 3-h extraction, the cap has fallen off the tube and both the cap and tube look dry, add up to 20 µL of 1X TE buffer to the cap and proceed with the experiments; the DNA is still there.

4. The film on the cap may fall into the extraction buffer. This occurs rarely since the film does not easily come off the cap. With the film in the extraction buffer, one gets a higher yield of DNA.
5. Add the 480-µL extraction buffer to the 20-µL aliquot of Proteinase K on ice and then distribute the 50-µL complete extraction buffer to tubes as they sit on ice immediately before you are ready to do the extraction. One must carefully preserve the activity of the Proteinase K.
6. DO NOT BOIL the samples after the extraction step is complete. One is more likely to lose the sample than to gain from inactivating the Proteinase K.
7. Use a Proteinase K that is heat labile so one has confidence that the activity of the Proteinase K has decreased after 3 h and will not interfere with subsequent experiments.
8. Test the DNA extraction efficiency before beginning the LCM. Section some test tissues onto the same glass slides that will be used for LCM. Stain the sections as one would normally for LCM, but do not coverslip the slides. Pipet 50 µL of the extraction buffer directly on the sections and pipet the cells off the slides into a sterile 0.5-mL centrifuge tube. Proceed with the extraction step as outlined above. This test sample can be used to test subsequent PCR.

4.3. PCR of DNA Derived From LCM

1. Use Taq Gold™ polymerase or another heat stable polymerase; the enzyme may need to be active through 60 cycles of PCR.
2. Thaw the LCM extracted DNA aliquot on ice, pipet the sample quickly into the PCR tube containing your PCR mix and immediately put the sample back in the −20ºC freezer. Use a styrofoam box or other container to protect the samples from freeze/thaw cycles if one uses a self-defrosting freezer. Avoiding the freeze/thaw cycles will preserve the samples for at least 6 mo.
3. The samples must be kept at −20ºC to keep the DNA intact. DNA after extraction was stored at 4ºC, −20ºC, and −80ºC. After 3 d at 4ºC, DNA was completely degraded. At −20ºC and −80ºC, DNA was stable for up to 6 mo.
4. Make serial dilutions of the "test sample" DNA extraction where the concentration of the DNA ranges from 1.0 pg to 500 ng. Perform PCR for one round of 60 cycles and 2 rounds of 30 cycles at several dilutions to determine which program gives optimal PCR results (*see* **Table 3** and p. 298 for best conditions). One can determine the lowest concentration of DNA, and therefore the lowest number of cells necessary, to get sufficient PCR product for further analysis.
5. Cells have been successfully microdissected from paraffin-embedded blocks and the DNA extracted and amplified. Sequencing was compared from frozen and paraffin-embedded tissue that had been microdissected and processed in the same manner. Sequences from the frozen tissues were clean and very readable. Sequence from the paraffin-embedded tissues had high background and appeared to have many mutations that were not seen with the frozen tissue. This finding has been reported by others ***(23)***.

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References

1. Jemal, A., Thomas, A., Murray, T., and Thun, M. (2002) Cancer statistics. *Ca. Cancer J. Clin.* **52,** 23–47.
2. Feldman, B. J. and Feldman, D. (2001) The development of androgen-independent prostate cancer. *Nature Rev.* **1,** 34–35.
3. Veldscholte, J., Ris-stapler, C., Kuiper, G. G. J. M., Jenster, G., Berrevoets, C. A., Claassen, E., et al. (1990) A mutation in the ligand binding domain of the androgen receptor of human LNCaP cells affects steroid binding characteristics and response to anti-androgens. *Biochem. Biophys. Res. Commun.* **173,** 534–540.
4. Harris, S. E., Harris, M. A., Rong, Z. X., Hall, J. A., Judge, S., French, F. S., et al. (1991) Androgen regulation of HBGF-1(aFGF) mRNA and characterization of the androgen receptor mRNA in the human prostate carcinoma cell line LNCaP/A-DEP, in *Molecular and Cellular Biology of Prostate Cancer* (Karr, J. P., Coffey, D. S., and Smith, R. G., eds.), Plenum, New York, pp. 315–330.
5. Tilley, W. D., Buchanan, G., Hickey, T. E., and Bentel, J. M. (1996) Mutations in the androgen receptor gene are associated with progression of human prostate cancer to androgen independence. *Clin. Cancer Res.* **2,** 277–285.
6. Takahashi, H., Furusato, M., Allsbrook, W. C. Jr., Nishii, H., Wakui, S., Barrett, J. C., and Boyd, J. (1995) Prevalence of androgen receptor gene mutations in latent prostatic carcinomas from Japanese men. *Cancer Res.* **55,** 1621–1624.
7. Suzuki, H., Sato, N., Watabe, Y., Masai, M., Seino, S., and Shimazaki, J. (1993) Androgen receptor gene mutations in human prostate cancer. *J. Steroid Biochem. Mol. Biol.* **46,** 759–765.
8. Elo, J. P., Kvist, L., Leinonen, K., Isomaa, V., Henttu, P., Lukkarinen, O., and Vehko, P. (1995) Mutated human androgen receptor gene detected in a prostatic cancer patient is also activated by estradiol. *J. Clin. Endocrinol. Metab.* **80,** 3494–3500.
9. Newmark, J. R., Hardy, D. O., Tonb, D. C., Carter, B. S., Epstein, J., Isaacs, W. B., et al. (1992) Androgen receptor gene mutations in human prostate cancer. *Proc. Natl. Acad. Sci. USA* **89,** 6319–6323.
10. Culig, Z., Hobisch, A., Cronauer, M. V., Cato, A. C. B., Hittmair, A., Radmayr, C., et al. (1993) Mutant androgen receptor detected in an advanced-stage prostatic carcinoma is activated by adrenal androgens and progesterone. *Mol. Endocrinol.* **8808–8809,** 1541–1550.
11. Taplin, M. E., Bubley, G. J., Shuster, T. D., Frantz, M. E., Spooner, A. E., Ogata, G. K., et al. (1995) Mutation of the androgen-receptor gene in metastatic androgen-independent prostate cancer. *N. Engl. J. Med.* **332,** 1393–1398.
12. Kleinerman, D. I., Troncoso, P., Pisters, L. L., Sleddens, H. M. E. B., Navone, N. M., Ordonez, van der Kwast, T. H., et al. (1994) Evaluation of androgen receptor expression and structure in bone metastases of prostate carcinoma. *Basic and clini-*

cal aspects of prostate cancer: An AACR Special Conference, Rancho Mirage CA, Dec. 8–12 (A-22).
13. Ruizeveld de Winter, J. A., Janssen, P. J., Sleddens, H. M., Verleun-Mooijman, M. C., Trapman, J., Brinkmann, A. O., et al. (1994) Androgen receptor status in localized and locally progressive hormone refractory human prostate cancer. *Am. J. Pathol.* **144,** 735–746.
14. Gaddipati, J. P., McLeod, D. G., Heidenberg, H. B., Sesterhenn, M., Finger, M. J., Moul, J. W., and Srivastava, S. (1994) Frequent detection of codon 877 mutation in the androgen receptor gene in advanced prostate cancers. *Cancer Res.* **54,** 2861–2864.
15. Evans, B. A. J., Harper, M. E., Daniells, C. E., Watts, C. E., Matenhelia, S., Green, J., and Griffiths, K. (1996) Low incidence of androgen receptor gene mutations in human prostatic tumors using single strand conformation polymorphism analysis. *Prostate* **28,** 162–171.
16. Kleinerman, D. I., Troncoso, P., Pisters, L. L., et al. (1996) Expression and structure of the androgen receptor in bone metastases of hormone refractory prostate cancer. *J. Urol.* **155,** 626A.
17. Peterziel, H., Culig, Z., Stober, J., Hobisch, A., Radmayr, C., Bartsch, G., et al. (1995) Mutant androgen receptors in prostatic tumors distinguish between amino-acid-sequence requirements for transactivation and ligand binding. *Int. J. Cancer* **63,** 544–550.
18. Tan, J.-A., Sharief, Y., Hamil, K. G., Gregory, C. W., Zang, D.-Y., Sar, M., et al. (1997) Dehydroepiandrosterone activates mutant androgen receptors expressed in the androgen dependent human prostate cancer xenograft CWR22 and LNCaP cells. *Mol. Endocrinol.* **11,** 450–459.
19. Emmert-Buck, M. R., Bonner, R. F., Smith, P. D., Chaquil, R. F., Zhuang, Z., Goldstein, S. R., et al. (1996) Laser Capture Microdissection. *Science* **274,** 998–1001.
20. Rubin, M. A. (2001) Use of laser capture microdissection, cDNA microarrays, and tissue microarrays in advancing our understanding of prostate cancer. *J. Pathol.* **195,** 80–86.
21. Goldstein, S. R., McQueen, P. G., and Bonner, R. F. (1998) Thermal modeling of Laser Capture Microdissection. *Appl. Opt.* **37,** 7378–7391.
22. Lubahn, D. B., Joseph, D. R., Sar, M., Tan, J.-A., Higgs, H. N., Larson, R. E., et al. (1988) The human androgen receptor: complementary deoxyribonucleic acid cloning, sequence analysis and gene expression in prostate. *Mol. Endocrinol.* **2,** 1265–1275.
23. Williams, C., Ponten, F., Moberg, C., Soderkvist, P., Uhlen, M., Ponten, J., et al. (1999) A high frequency of sequence alterations is due to formalin fixation of archival specimens. *Am. J. Pathol.* **155,** 1467–1471.

22

Clonality Analysis by T-Cell Receptor γ PCR and High-Resolution Electrophoresis in the Diagnosis of Cutaneous T-Cell Lymphoma (CTCL)

Ansgar Lukowsky

Abstract

During T-cell maturation, T-cell receptor (TCR) gene segments rearrange, resulting in a new, unique DNA configuration. The recombined TCR gene loci display a high degree of nucleotide sequence variability. Molecular biological clonality assays focus on this cell-specific DNA pattern. The finding of an identical TCR rearrangement in a large number of T lymphocytes signals a malignant proliferation, although clonality is not always equivalent to malignancy. Thus, detection of clonal TCR γ rearrangements by polymerase chain reaction (PCR), followed by high-resolution electrophoresis is a valuable tool in the diagnosis of cutaneous and other T-cell lymphomas. For the clonality assay described here, all rearrangements of T cells present in a given sample are amplified by a set of only three TCRγ-PCRs. The products are investigated by either heteroduplex temperature gradient gel electrophoresis (HD-TGGE) or fluorescent fragment analysis (FFA) on a capillary DNA sequencer (or by both methods), for clonality. Both electrophoresis techniques show highly reproducible results and are comparatively easy to conduct, however, specific instruments are required. Concerning lower detection thresholds, the methods need a minimum of about 1% of clonal T-cells in mixtures with polyclonal T-cells for revealing clonality.

Key Words: Lymphoma; T-cells; clonality; PCR; high resolution electrophoresis.

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1. Introduction

T-lymphocyte subsets express T-cell receptors (TCR) on their cell surface, each molecule consists of two out of four different peptide chains designated as α/β or γ/δ TCR. The germ line configuration of each TCR locus (α, β, γ, δ) comprises different numbers of separate Variable (V), Joining (J), and Constant (C) gene elements, β and δ loci additionally have Diversity (D) segments. During T-cell maturation, each locus rearranges distinct V, D (only in TCRβ and δ), and J gene segments resulting in a new DNA configuration. The recombined TCR genes display a high degree of nucleotide sequence variability, created by fusion of differently selected V, D, J segments (combinatorial diversity) and by a random insertion or deletion of bases at the V-J, V-D, or D-J boundaries, which results in the formation of so-called junctional or N-regions of template independend nucleotides, exhibiting a high degree of sequence variety (junctional diversity). In conclusion, every T cell carries an individual genomic DNA pattern known as TCR rearrangement, as is depicted for the TCRγ locus in **Fig. 1**. Molecular biological clonality assays focus on the individual DNA pattern of every T cell: The demonstration of an identical TCR rearrangement in a number of T lymphocytes signals a malignant proliferation named T-cell lymphoma, although clonality does not necessarily mean malignancy. T-cell lymphomas represent approx 80% of all skin associated lymphoma cases ***(1)***. In these cutaneous T-cell lymphomas (CTCL), clonality assays are useful for differentiation between CTCLs, particularly of initial stage and benign inflammatory skin lesions ***(2)***. Early CTCL and inflammatory dermatoses are often difficult to distinguish, as, in many cases, the phenomenological behavior of the neoplastic T cells initially resembles that of reactive T lymphocytes. Thus, the detection of clonal T cells in cutaneous infiltrates confirms the diagnosis of a CTCL. In mycosis fungoides (MF), the CTCL prototype, up to 90% of lesional skin biopsy samples carry clonal T cells detectable by various PCR analyses ***(2)***. Clonal T-cells corresponding to the cutaneous clone have also been detected in the peripheral blood of most patients with MF ***(3)***.

Numerous assays have been developed to detect clonality of T cells. First, Southern blot analysis, displaying TCR mediated diversity of the restriction fragment length, has been applied. However, this method is quite laborious, technical artifacts occur frequently and an amount of at least 5–10 µg purified high molecular genomic DNA is required. Therefore, this method cannot be applied for paraffin embedded specimens or small skin punch biopsy samples. Thus, PCR-based methods, being far more useful, have been developed. Comparing the four TCR gene complexes, only the TCRγ locus is well suited for diagnostic PCR, as it consists of only four V gene families with 16 gene segments and five J gene segments ***(4)***, which can all be covered by a small number of primers.

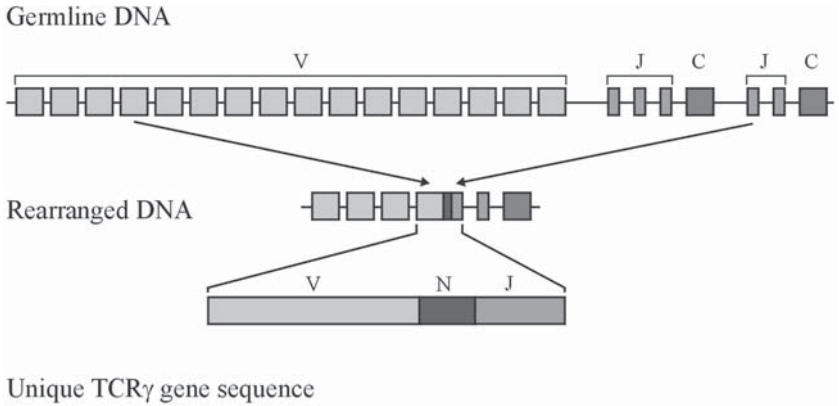

Fig. 1. Schematic pattern of TCRγ gene rearrangement.

Apart from very rare exceptions, TCRγ chains are not expressed on malignant T-cells in CTCL, but the clonal cells bear an individual TCRγ gene rearrangement that has been created in early T-cell maturation. In TCRγ -PCR based methods, rearrangements of all present T cells are amplified and the products are investigated by high-resolution electrophoresis for presence of multiple identical, i.e., clonal amplificates, as can be seen in **Figs. 2** and **3** for both electrophoresis techniques described here. The diagnostic sensitivity of TCRγ-PCR depends on the fraction of V and J segments recognized by the primers used. No consensus primers encompassing all V or J segments, respectively, have been found. Multiplex PCR may provide the amplification of all rearrangeable segments, but primers always possess different priming efficiencies and binding sites. These differences severely affect the analysis of PCR products. For example, length differences of the PCR products, additional to those produced by the N-region, may result, and additional bands on electrophoresis gels can occur, which lead to misinterpretations. For the largest Vγ gene family VγI, which consists of eight gene segments (V1-8), appropriate consensus primers have been derived. By PCR amplifying rearrangements of V1-8 and J1/J2 gene segments by a single pair of consensus primers, clonality can be detected in about 70% of CTCL skin biopsies ***(5)***, because of nonrandom V- and J-gene segment usage. An optimal TCRγ PCR assay should cover the TCRγ gene segments V1-8, as well as V9, V10, and V11 belonging to the Vγ families II-VI, in combination with J1, J2, JP1, and JP2. Rearrangements using the γ-pseudogenes VA, VB, V12, and JP are very rare or do not occur, thus, corresponding primers can be omitted in TCRγ-PCR, without decreasing diagnostic sensitivity. Consequently, a set of three assays is recommended: PCR-1 with primers for V1-8 and J1/J2, PCR-2

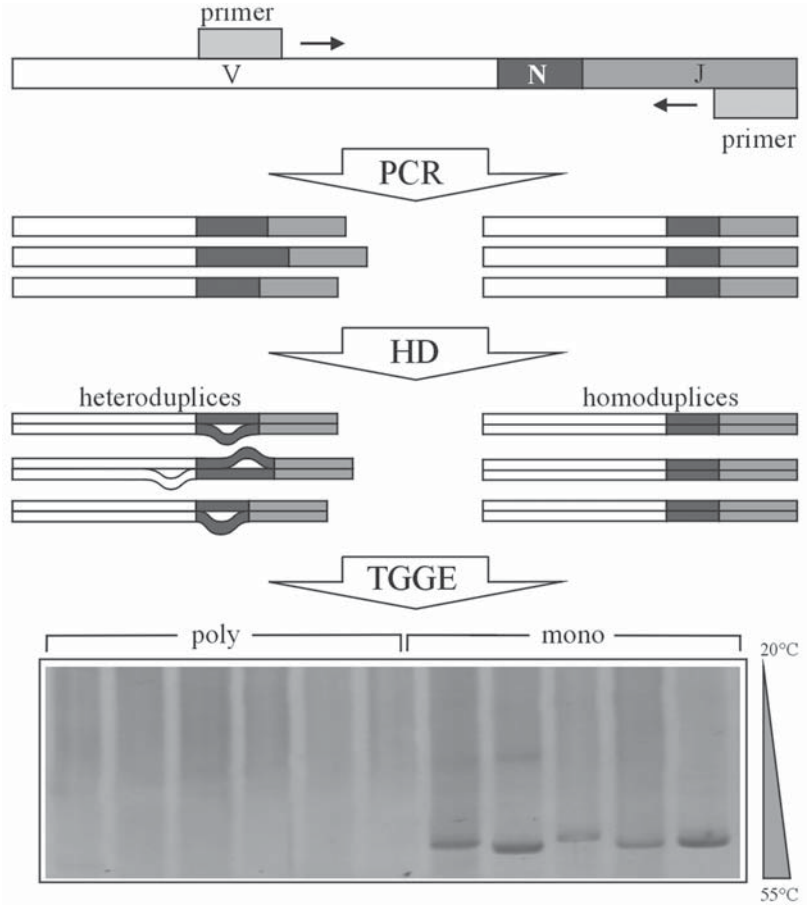

Fig. 2. Principles of TCRγ-PCR with subsequent HD-TGGE.

with primers for V9, V10, V11, and J1/J2 and PCR-3 with primers for V1-8, V9, V10, V11, and JP1/JP2. However, even if PCR covering all possible V-J combinations are performed, detection of clonal T cells fails in at least 10% of lesional skin samples in CTCL ***(2)***, possibly because of a low number of clonal cells in the sample, chromosomal aberrations, or, as an exception, by TCRγ germ line configuration. In addition to PCR, the electrophoresis method applied for product analysis determines diagnostic sensitivity, detection threshold, and specificity of the assay. Following amplification of TCRγ rearrangements of all T cells, present in the processed sample, high-resolution electrophoresis should differentiate between polyclonal and monoclonal products. Sophisticated DNA

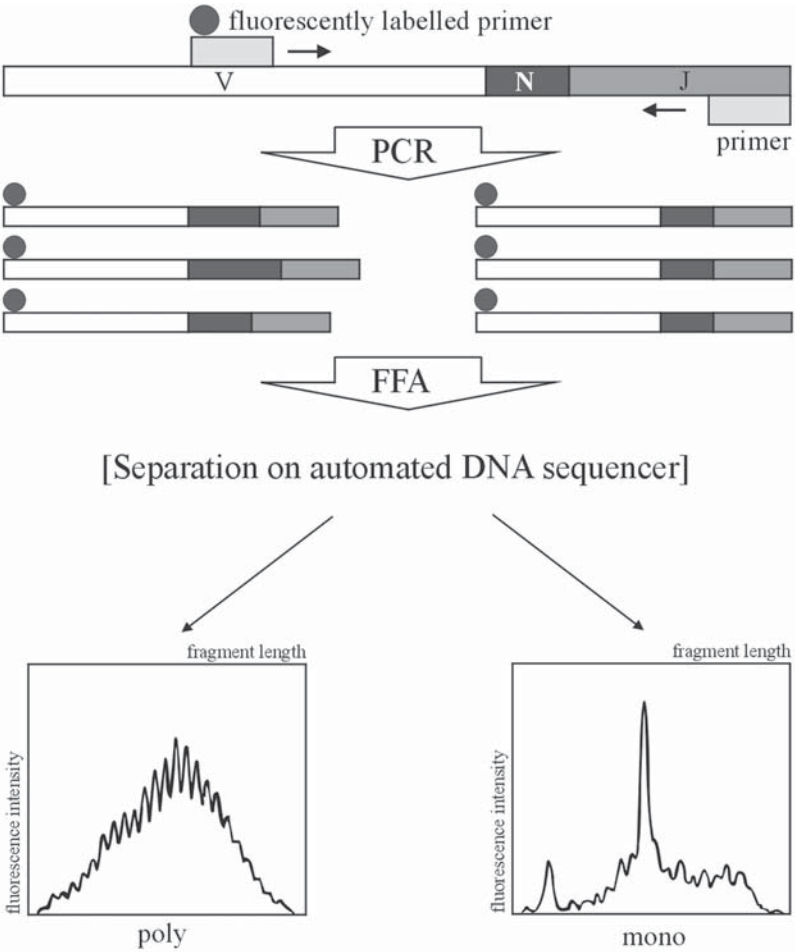

Fig. 3. Principles of TCRγ-PCR with subsequent FFA.

electrophoresis techniques, in particular, denaturing gradient gel electrophoresis (DGGE) ***(6)***, temperature gradient gel electrophoresis (TGGE) ***(7–9)***, single-strand conformation polymorphism electrophoresis (SSCP) ***(10,11)***, and fluorescent fragment analysis (FFA) on automated DNA sequencers ***(5,9,11–17)***, have been utilized in this context. The methods suggested here, i.e., heteroduplex (HD)-TGGE and FFA, offer convincing advantages. Both methods provide reproducible results and are comparatively easy to conduct, once established. HD-TGGE allows for a simple preparation of the separated clonal product for establishing its DNA sequence. FFA provides an accurate estimation of PCR product lengths, which helps to overcome false interpretations or to identify contaminations and

permits for an immediate comparison of clonal rearrangements from different samples. FFA separations run automatically, require no staining procedures and include online data processing. However, both techniques require specific instruments. Concerning sensitivity, a minimum of at least 1% of clonal T cells in mixtures with polyclonal T cells is required for detection by FFA as well as by HD-TGGE *(5,18)*.

2. Materials

Unless indicated otherwise, use only molecular biology grade or ultrapure chemicals and reagents. Warning: The chemicals acrylamide, N,N´-methylenbis-acrylamide, N,N,N'N'-tetramethylethylenediamin (TEMED), formamide, acetic acid, $AgNO_3$, NaOH, and formaldehyde are toxic and/or caustic substances, handle always with precautions recommended, wear protective eyewear, clothing and gloves. For detailed information obtain copies of the Material Safety Data Sheet (MSDS) from suppliers.

2.1. Preparation of Genomic DNA

1. Ethanol, GR (for analysis).
2. Xylene, GR, or xylene-substitute (*see* **Note 1**).
3. Proteinase K, prepare stock solution of 10 mg/mL, keep at 4ºC or at –20ºC in aliquots.
4. DNA extraction buffer: 10 m*M* Tris-HCl pH 8.3, 50 m*M* KCl, 0.45% Nonidet-P40, 0.45% Tween-20.

2.2. PCR Reagents

1. ddH_2O, HPLC grade.
2. 10X PCR buffer (GeneAmp10X PCR buffer II, Applied Biosystems, Forster City, CA).
3. 25 m*M* $MgCl_2$ (supplied with *Taq* polymerase).
4. *Taq* polymerase (AmpliTaq DNA Polymerase, Applied Biosystems).
5. dNTP-mix from100 m*M* dNTPs stocks (Ultrapure dNTP set Amersham-Pharmacia Biotech Inc., Piscataway, NJ), make 2.5 m*M* solution by adding 25 µL of each dNTP to 900 µL ddH_2O.
6. 50 µ*M* stock of each primer oligomer (*see* **Table 1**), with the dye 5-carboxyfluorescein (FAM) 5'-fluorescently-labelled primers VG1, JG1/2 or JGP1/2, respectively, in case of subsequent FFA.

2.3. HD-TGGE

1. 30% acrylamide/*bis*-acrylamide stock solution: 30 g/ 0.5 g/ 100 mL dd H_2O, store at 4ºC in dark.
2. TEMED.
3. 4% ammonium persulphate, store at –20ºC in 0.5-mL aliquots.
4. Urea, GR.

Table 1
Sequences of PCR Oligonucleotid Primer

Name	Primed gene segments	Position	5'-3' sequence
VG1*	Vγ 1, 2, 3, 4, 5, 5p, 6p, 7p, 8	104-121	CTACATCCACTGGTACCT
VG1int	Vγ 1, 2, 3, 4, 5, 5p, 6p, 7p, 8	136-153	AGRCCCCACAGCATCTTC
VG9	Vγ 9	121-138	ATTGGTATCGAGAGAGAC
VG9int#	Vγ 9	188-208	GGAAAGGAATCTGGCATTCCG
VG10/11	Vg10, 11, B, (A)	111-129/ 117-135	CACTGGTACKKGCAGAAAC
VG10int#	Vg10	180-198	CCGCAGCTCGACGCAGCA
VG11int#	Vg11	175-195	GCTCAAGATTGCTCAGGTGGG
JG1/2*	Jg1, 2	27-44	CAACAAGTGTTGTTCCAC
JGP1/2	JgP1, P2	31-48	CTATGAGCYTAGTCCCTT

*, # according ***(23)*** and ***(24)***, modified.

5. 40% glycerol.
6. 1 *M* MOPS, adjusted with NaOH to pH 8.0.
7. 10X loading buffer: 0.25% bromophenol blue, 0.25% xylene xyanol, 15% Ficoll.
8. 0.1% Triton X-100.
9. DNA molecular weight marker V (Roche Applied Science, Mannheim, Germany).
10. TGGE maxi Gel support films (Whatman Biometra, Goettingen, Germany).
11. Acryl-Glide™ (Amresco Inc., Solon, OH).

2.4. Silver Staining Reagents

Ensure that ddH_2O and chemicals (GR for analysis) are free from chloride ions which impair severely silver staining, causing a dark brown or even black background.

1. Fixative: 10% ethanol, 0.5% acetic acid.
2. Silver staining solution: 0.1% $AgNO_3$, reusable for up to 10 stainings, dilute from a stock solution of 1%, store both silver solutions in the dark.
3. Developer: 1.5% NaOH, 0.01% $NaBH_4$, 0.015% formaldehyde (2.4 mL of 37% formaldehyde stock per 600 mL developer), prepare solution freshly just before staining.
4. Stop solution: 0.75% Na_2CO_3.

2.5. FFA on ABI 310 PRISM™

1. Formamide, deionized (pH 7.0–9.0), stored in 0.5-mL aliquots at –20ºC for up to 4 mo.
2. Reagents and consumables: GeneScan-500™ TAMRA size standard, ABI Prism™ buffer 10X with ethylenediamine tetraacetic acid (EDTA), POP-6™ (Performance

Optimized Polymer 6), capillaries 47 cm × 50 µm, 0.5-mL sample tubes with septa (Applied Biosystems, Foster City, CA).

2.6. Instruments

1. Thermal cycler for PCR, preferentially with heated lids.
2. TGGE Maxi System, Whatman-Biometra®, Goettingen, Germany.
3. DNA-Sequencer ABI 310 PRISM with GeneScan 672 software (Applied Biosystems).

3. Methods

3.1. Preparation of DNA (see Note 2)

3.1.1. Preparation from Paraffin-Embedded Tissue

Carry out all steps at room temperature.

1. Cut about 10 sections of 10-µm thickness per biopsy sample, collect the sections in a 1.6- or 2-mL centrifuge tube.
2. Add 1 mL of xylene (or xylene-substitute), vortex shortly and centrifuge for 5 min at approx 10,000–13,000*g* in a bench top microcentrifuge, remove the supernatant carefully with a pipet, but as completely as possible, repeat xylene extraction with the pellet in the same manner.
3. Extract the pellet twice with 1 mL of 96% or absolute ethanol in the same way and dry the pellet thoroughly by vacuum (exiccator). The resulting pellet should look powder-like and no longer smell of ethanol.
4. Add 200 µL DNA extraction buffer and 20 µL proteinase K stock solution, vortex thoroughly and incubate overnight at 55ºC, if the pellet is small (i.e., only just visible at the tube bottom) reduce the volume of buffer/proteinase K solution to 100/10 or 50/5 µL.
5. Incubate for 15 min at 95ºC, centrifuge for 10 min at about 13,000*g* and pipet the supernatant into a new sterile sample tube, keep in a refrigerator for PCR analysis up to one month. For longer storage freezing at –20ºC is recommended.

3.1.2. Preparation from Frozen Tissue

Start at **step 3** (above) with 10 cryo-sections of 10 µm each. The sections should be kept frozen until the DNA extraction starts.

3.1.3. Preparation from Peripheral Blood Mononuclear Cells (PBMC), Isolated T-cell Subfractions and T-cell Cultures

Harvest cells from 10 mL blood by standard ficoll density gradient centrifugation, wash twice with PBS, sediment cells (approx 10^7 cells) and start at **step 3** (above). Reduce volume of buffer/proteinase K solution proportionally with fewer cells. Proceed with T-cell subfractions or T-cells grown in culture in the same way (*see* **Note 3**), always wash cells twice with PBS following sedimentation from culture medium.

3.2. TCRγ-PCR (see Note 4)

1. Adhere to general advices for PCR performance ***(19)***.
2. Apply three PCR amplifying rearrangements of the TCRγ gene segments V1-V11 with J1, J2 or JP1, JP2, respectively, *see* **Table 1** for primer sequences. The primer combinations are:
   PCR-1 (consensus PCR): VG1 and JG1/2. For FFA one of both primer FAM-labeled.
   PCR-2 (multiplex PCR): VG9, VG10/11 and JG1/2. For FFA JG1/2 FAM-labeled.
   PCR-3 (multiplex PCR): VG1, VG9, VG10/11 and JGP1/2. For FFA JGP1/2 FAM-labeled (*see* **Notes 5–6**).
3. Prepare a mix of 50 µL including
   a. 5 µL 10X PCR buffer.
   b. $MgCl_2$, final conc 0.4 m*M* for PCR-1, 0.25 m*M* for PCR-2 and -3.
   c. dNTP-mix, final conc 2 m*M* for each dNTP.
   d. PCR primers, final conc 0.67 µ*M* for PCR-1, 0.50 µ*M* for PCR-2 and -3.
   e. *Taq* polymerase, 3 U for PCR-1, 2 U for PCR-2 and -3.
   f. 2–5 µL DNA solution.
   g. Water to bring volume to a total of 50 µL (*see* **Notes 7–8**).
4. Prepare a master mix, distribute in 0.5-mL PCR tubes and add DNA solution last. Denature at 95ºC for 5 min, perform 40 cycles of denaturation at 94ºC, annealing at 58ºC, extension at 72ºC, 1 min for each step and add one final extension of 4 min at 72ºC. Prolong final extension for 45 min in PCR with the fluorescently labeled primers for subsequent FFA. Screen with 6–8 µL of each reaction for successful amplification of 240–260 bp fragments on a 2% ethidium bromide stained agarose gel high resolution electrophoresis (*see* **Notes 9–10**).

3.3. HD-TGGE

HD-TGGE requires an instrument forming the temperature gradient such as the commercially available TGGE Maxi System of Whatman-Biometra. The present protocol refers to this system. In most parts the procedure is performed in accordance with the manufacturer's standard protocol, which is described in: Whatman-Biometra TGGE Maxi System manual, version 3.02, October 2000.

3.3.1. Gel Casting

Cast a standard gel for parallel TGGE, containing 30–32 slots, 8% of polyacrylamide gel for MOPS buffer system (see also manual, p9 ff). Carry out all steps at room temperature.

1. Assemble the gel pouring cuvette (*see* **Note 11**): Lay the gel support film on the cover glass plate (plain) and attach it by careful rubbing and pressing with a wipe, so that it is fixed evenly on the whole plate. Put the glass plate with spacers and slot formers upwards and lay the U-shaped silicone rubber sealing strip outside around the spacer. Attach the cover plate with the film inwards and fix with three clamps on each side placed on the spacers or seal, respectively. Set the gel cuvet

vertical, the open side upward. Do not let the assembly stand only on the lower three clamps, fix additionally to prevent falling.
2. Prepare the gel solution. For the gel a urea concentration of 8 *M* instead of 7 *M* is preferred, therefore to a 50-mL gel mixture 24 g solid urea (instead of 21 g) are added. Pour the gel slowly trying to prevent air bubbles, but bubbles can be removed during or immediately after pouring using a narrow, long, clean strip cut from a gel film being always on hand before pouring. Pour the gel through a 50-mL syringe with a wide needle, fixed directly over the open site of the cuvet.
3. Let the gel polymerize for 2 h, do not move or disturb the gel assembly during polymerization. Prevent gel from drying because of longer exposure to air, leave gel in the assembly until applied for electrophoresis, i.e., sample preparation is done. Keep the gel at room temperature, usage on the same day is recommended.
4. For disassembling first remove the clamps and then the silicon sealing and the cover plate. Slowly peel the support film containing the gel from the other glass plate, begin with a corner of the bottom, be aware that the gel adheres to the film and the slots will not be damaged. Set up gel for electrophoresis on the thermoblock as outlined in the manual.

3.3.2. Sample Preparation, Loading, and Electrophoresis

1. For heteroduplex formation add 0.5 µL loading buffer to 10 µL of each PCR product heat for 10 min at 95ºC in a thermocycler then for a further 20 min at 55ºC, lower temperature with a ramping rate of about 6ºC/min.
2. Load immediately 5–6 µL of each sample on the gel for parallel TGGE. Apply a molecular weight marker in one or both outer slots: mix 1-2 µL marker with 3 µL 1XMOPS and 0.5 µL loading buffer and load without heteroduplex formation. Use a temperature gradient from 20º to 55ºC.
3. Perform pre-run and temperature equilibration steps as described, followed by main run at maximally 400 V not exceeding 25–30 mA.
4. Stop separation if the bromophenol blue front just reaches the anode wick, taking approx 2.5 h, remove the gel from the device and continue at once with staining.

3.3.3. Silver Staining (see **Note 12**)

Always wear powder-free clean gloves, hold gel only at the support film, do not touch or press the gel surface, gently agitate during all incubations. Carry out all steps at room temperature.

1. Put the gel, support film facing downwards, in a plastic or glass tray large enough to allow some movement by agitating. Rinse shortly with approx 200 mL ddH_2O water to wash off adhering thermal coupling solution and decant.
2. Incubate twice with 300 mL fixer solution for 3 min each and discard.
3. Incubate with 300 mL silver staining solution for 10–15 min and pour solution back into the bottle, it can be reused at least five times.
4. Wash shortly twice (approx 30 s each wash) with approx 300 mL ddH_2O.

5. Wash shortly with 200 mL developing solution, decant as soon as a dark precipitate occurs, add again 300 mL developer and incubate for about 30 min. It is useful to attend the gel and proceed with **step 6**, if the DNA bands do not get darker, although, with high water quality and well-prepared solutions overstaining very rarely occurs.
6. Add stop solution, incubate for 10 min, decant, and rinse shortly with ddH_2O.
7. Let the gel air-dry until no fluid is visible on the gel surface.
8. Examination of gel on a white-light table: polyclonal amplification products form DNA double strands with mismatches in the N region, decreasing the thermal stability of the N region and, thus, altering the fragment migration in the temperature gradient. In consequence, a more or less visible broad smear appears in the lane. In contrast, clonal products produce more stable homoduplices, which migrate as sharp, strongly stained bands into the correct size range of about 240–260 bp. Disregard nonspecific bands, which are stained more weakly and appear repeatedly in the same position in different samples. Nonspecific bands are generated mostly from DNA of PBMC, i.e., from high molecular template. A silver-stained HD-TGGE gel is shown with **Fig. 4**.
9. Following evaluation or photography, seal the gel in a plastic bag, Tightly sealed gels can be stored for up to 5 yr.

3.3.4. Re-PCR and Seminested PCR for Confirming Results of Clonality Detection by DNA Sequencing

1. Cut out the clonal band with a disposable scalpel, transfer to a sterile tube, add 20–40 µL PCR buffer and agitate overnight at room temperature.
2. Centrifuge for approx 30 s at 10,000*g* and perform re-amplification with 2–5 µL of the supernatant, as outlined above, use also the same primer sets.
3. If no products can be obtained, apply internal V-primers for re-PCR: Substitute VG1 by VG1int, VG9 by VG9int, VG10/11 by VG10int and VG11int (both primers mixed or each in a separate assay), respectively. PCR products of about 210–230 bp with VG1int and of about 170–200 bp with VG9int, VG10int, and VG11int are generated.
4. Following purification by an appropriate kit the amplificates can be applied for direct DNA sequencing.

3.4. FFA (GeneScan Analysis) on ABI 310 PRISM (see Note 13)

FFA on an automated DNA sequencer is based only on size differences of DNA amplicons, however, because of high resolution a one base pair exact size determination of each PCR product is provided. Separation and GeneScan Analysis on the ABI 310 PRISM instrument requires four different software files: 1) run modules to process a sample; 2) size standard files for computing the length of the products separated; 3) analysis parameter files for data analysis; and 4) matrix files for correcting overlap of dye spectra. The module files

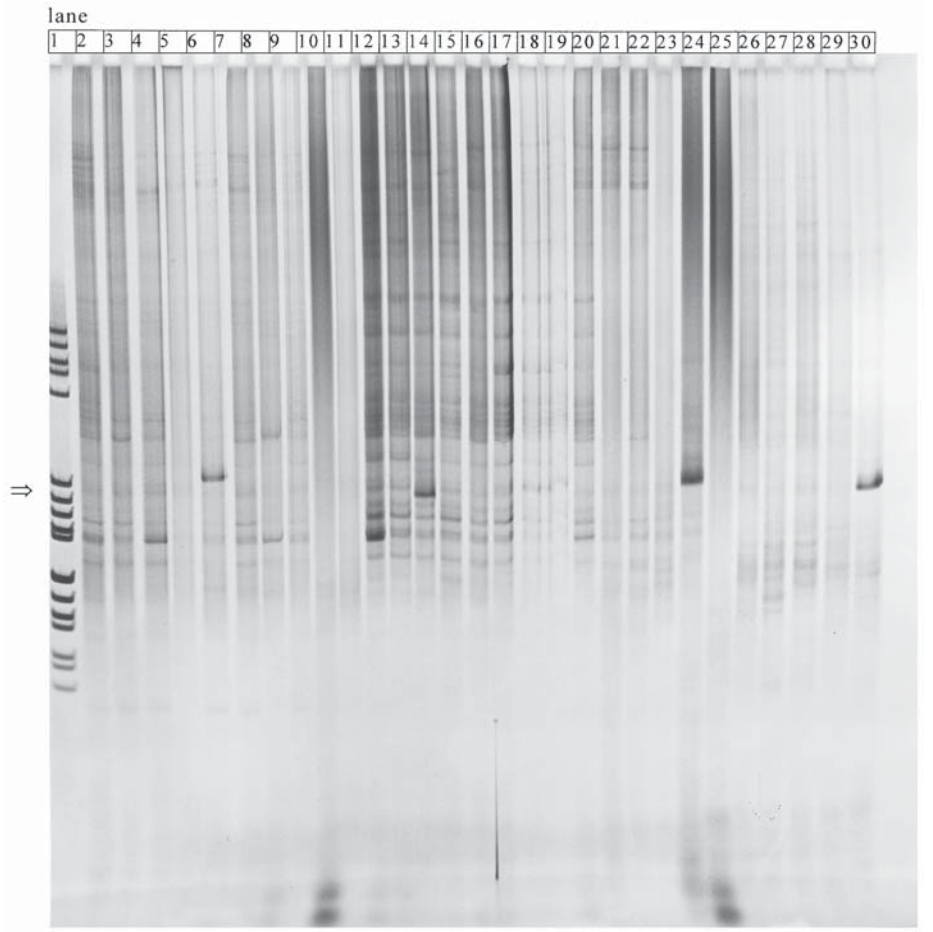

Fig. 4. Photography of a silver-stained HD-TGGE gel. Lane 1, size marker; lanes 12–20, blood samples; lane 24, Jurkat cells (clonal control); remaining lanes, paraffin-embedded skin samples. Clonality is seen in lanes 6, 14, 24, and 30. Arrow: approx. position of clonal products.

are selected for the run as part of the Data Collection program of the machine (*see* ABI PRISM 310 Genetic Analyzer user's manual). The other three types of files have to be generated (see instruments manual and ABI PRISM GeneScan Analysis software user's manual).

1. Prepare the instrument as described in the manual, but regard that polymer POP-6 is used for GeneScan separation, a matching run module must be selected or created (*see* **Note 14**). Finally, preheat the capillary to 60ºC to save time.

2. Mix 12 µL deionized formamide, 0.5 µL GeneScan-500 TAMRA size standard, and 1 µL of the PCR amplificate in the sample tube and cover with septa. Vortex shortly and spin down. Do not forget to label the tubes.
3. Heat for 5 min at 95ºC in a thermoblock. If a thermocycler with heated lids is used, switch off the lid and do not close to avoid melting or sticking of the septa at the lid. Chill immediately on ice to produce single stranded DNA, hold on ice until loading in the autosampler.
4. Open the data collection program, and write GeneScan sample sheet and injection list containing the sample names and injection order of the run. Choose a 5–15 s injection time, depending on the intensity of the clonal band on the agarose gel, 36 min separation time in a 47 cm POP 6-filled capillary. Turn on GeneScan analysis, for convenience select autoanalysis.
5. Transfer samples tubes to the autosampler, and start running the injection list at 60ºC and 15 kV. The supplier quotes a maximal number of 100 separations per capillary. However, up to 300 runs are possible. The capillary should be replaced when peak-tailing, first visible at size marker peaks, occurs. It is important to prevent drying of the polymer-filled capillary. Make sure that sufficient electrophoresis buffer is in the buffer vial (position 1 on the autosampler). The capillary should always be immersed, thus check the level in the water containing vials in position 2 and 3 as well.
6. While running, check current regularly, it should be about 10 µA (range: 7–12 µA). Low or blocked current is most frequently caused by small air bubbles in the channel of the pump block, by loss of buffer in the glass vial in position one of the autosampler, or by upward shift of capillary, then adjust and tape the capillary again. Also check the raw data display. Raw electrophoresis curves without any peaks indicate a temporary interruption of current flow implying that no material has been injected. Moreover, the calibration settings of the autosampler may be incorrect and recalibration is necessary.
7. Following separation and analysis print the electropherogram showing relative fluorescence intensity on the y-axis and fragment size on x-axis (GeneScan profile). Print also all peak data for the size range expected, which is approx 239–261 bp for PCR-1, 233–256 bp for PCR-2, and 246–267 bp for PCR-3. You can already print the profile of each completed sample, during running the injection list. The presence of a clonal T-cell population is indicated if one or two peaks clearly dominate the fluorescence intensity profiles, as can be seen from **Fig. 5**. Occurrence of two dominant peaks shows a biallelic clonal rearrangement (*see* **Notes 15–16**). Disregard irregular formed profiles, showing not the 1 bp peak-distance as well as profiles with very low fluorescence intensities, i.e., less than 200 U for the highest peak.

4. Notes

1. Because xylene is toxic and an irritant, substitutes have been developed, which work equally well. The author now uses Roticlear® (Carl Roth GmBH&Co., Karlsruhe, Germany).

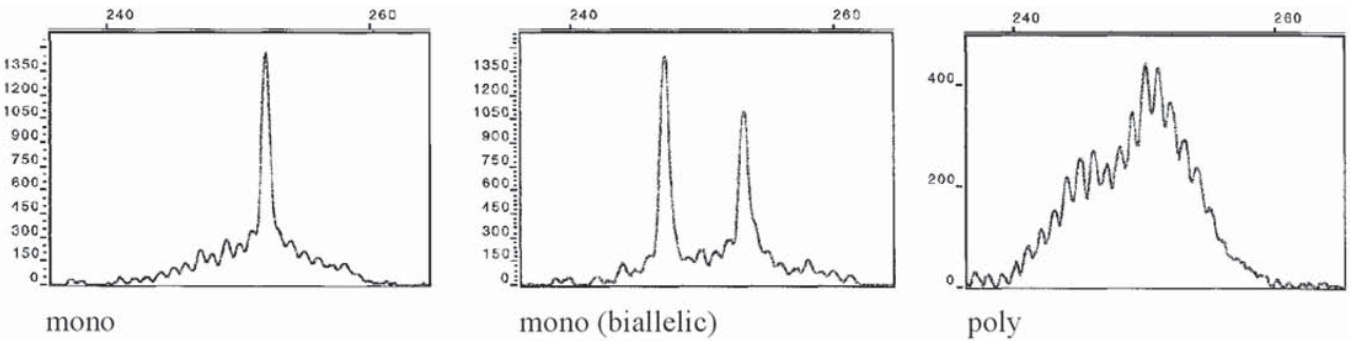

Fig. 5. Typical TCRγ-PCR FFA profiles: clonal, clonal-biallelic rearranged and polyclonal.

2. For PCR, the DNA preparation method described here serves its purpose, although it does not ensure the removal of all proteins. The DNA extract gained is a well-suited template for PCR amplification. Alternatively, commercial DNA isolation kits for the various materials in question can be used. In most instances purifications by these kits do not improve subsequent PCR results. In fact, the DNA quality for PCR depends largely on its source and the preanalytic treatment. For paraffin-embedded tissues, mainly available for analysis, the fixation time and the quality of the fixer, i.e., buffered formaldehyde-solution, are crucial.
3. A minimum of 10^4 cells, lysed in 50 µL DNA extraction buffer/5 µL proteinase K is suggested. Lower cell numbers may yield PCR products but sampling errors can cause false clonal results.
4. Necessity of control PCRs: the quality of the DNA samples can be evaluated by standard PCRs with primers specific for genomic DNA housekeeping genes, such as for a conserved region of the human β-globin gene ***(20)***, which the author occasionally applies. However, a successful β-globin gene PCR does not guarantee successful PCR for TCRγ-gene rearrangements, possibly due to the lower numbers of copies retained in the sample of interest. Varying PCR results are regularly observed in paraffin-isolated DNAs.
5. In CTCL, clonality is mostly found by PCR-1, i.e., in approx 70% of all lesional skin samples and only rarely by PCR-3 ***(5)***. Thus, workload can be reduced by sequentially running PCR-1 and (if polyclonal rearrangements only are seen) by PCR-2 and -3.
6. Include negative (no DNA) and clonal positive controls in each assay, as clonal cells, e.g., the long-time established T cell lines Jurkat in PCR-1,-2 and Molt-4 in PCR-3.
7. The PCR volume of 50 µL described here yields sufficient material for repeated electrophoresis, however, 25 µL works as well. The higher primer and $MgCl_2$ concentrations of PCR-1 improve amplification of paraffin-embedded DNA. *Taq* DNA polymerases other than AmpliTaq have been found to work equally well. The efficiency of TCRγ-PCR for DNA of paraffin embeddings was not improved considerably by usage of a hot-start *Taq* polymerase (AmpliTaq gold DNA Polymerase, Applied Biosystems).
8. Begin with 5 µL paraffin-derived DNA solution and repeat PCR with 2 µL, if no products are generated. Use 2 µL DNA from frozen or fresh tissue and PBMC. This amount should correspond to 0.1–1 µg.
9. Control screening on agarose gels is recommended in particular for amplifications of paraffin-isolated DNAs, because preparations of this source sometimes fail to yield products due to wrong fixation, for example.
10. Note that PCRs with the fluorescently labeled primers are less effective, probably as a consequence of sterical hindrance. Consequently, DNA samples of poor quality, i.e., from paraffin-embeddings fail more frequently in yielding a product sufficient for subsequent FFA as compared to non-labeled PCR. The use of fluorescently labeled nucleotides for the set of TCRγ-PCR described here, cannot be recommended, because it provides fuzzy FFA profiles, sometimes obscuring clonality.

11. Treatment of the glass plate with Acryl-Glide before *each* gel preparation, as described in the manufacturer's instruction, is unnecessary. It is sufficient to clean the plate thoroughly with a mild detergent and then with 70% ethanol. Application of Acryl-Glide or siliconization by 7% dichlorodimethylsilane should be repeated, if the gel tends to stick to the plate. Passing the gel solution through a sterile filter can also be omitted, although any precipitates or opacity must be avoided. Urea should be completely dissolved and the acrylamide/*bis*-acrylamide stock solution should also be clear.
12. A sufficiently sensitive silver staining procedure requiring less time, originally developed for TGGE by Qiagen ***(21)***, slightly modified, is recommended instead of the Biometra protocol.
13. Similarly, the FFA of TCRγ-PCR using the GeneScan software has been carried out on the automated gel type DNA sequencing instruments ABI 373A ***(14)*** and ABI 377 ***(11)***.
14. For the capillary sequencer ABI 310 PRISM originally the polymer POP-4 has been developed for FFA. However, using POP-4 requires a change of polymer in case of subsequent DNA sequencing and vice versa. Thus, for convenience POP-6 is suggested here for sequencing and GeneScan runs as well.
15. Polyclonal profiles may have or may not have a Gaussian shape, normal distribution of peak-heights is mostly received from PBMC. The occurrence of oligoclonal T-cell expansions in CTCL, showing more than two dominant peaks is still considered controversial ***(5)***. Note that oligoclonality may be also due to a partial DNA degradation or an inadequate DNA template input ("sampling error").
16. In order to discriminate with certainty between clonal and polyclonal rearrangements it is advisable to calculate the clonal peak-height ratio by dividing the clonal peak height by the mean height of left and right adjoining peaks ***(5)***. In clonal rearrangements the ratio should be greater than 2 in CTCL samples. Additionally, the height of the suspected clonal peak has to exceed the mean height of all polyclonal background peaks to avoid misinterpretations because of small isolated peaks at the edges of the profile. Use peak-heights above baseline as recorded by the GeneScan program for calculations. In case of two dominant peaks (representing a biallelic rearrangement) only the highest peak should be considered. At present there is no standard-calculation or -definition for distinguishing clonal from polyclonal peaks. Other calculation procedures of peak ratios for defining clonality relate the height of the suspected clonal peak to the height of the highest polyclonal background peak ***(16,22)***.

References

1. Willemze, R., Kerl, H., Sterry, W., Berti, E., Cerroni, L., Chimenti, S., et al. (1997) EORTC classification for primary cutaneous lymphomas: A proposal from the cutaneous lymphoma study group of the European Organization for Research and Treatment of Cancer. *Blood* **90,** 354–371.

2. Bachelez, H. (1999) The clinical use of molecular analysis of clonality in cutaneous lymphocytic infiltrates. *Arch. Dermatol.* **135,** 200–202.
3. Muche, J. M., Lukowsky, A., Asadullah, K., Gellrich S., and Sterry, W. (1997) Demonstration of frequent occurrence of clonal T cells in the peripheral blood of patients with primary cutaneous T cell lymphoma. *Blood* **90,** 1636–1642.
4. Arden, B., Clarke, S. P., Kabelitz, D., and Mak, T. W. (1995) Human T-cell receptor variable gene segment families. *Immunogenetics* **42,** 455–500.
5. Lukowsky, A., Richter, S., Dijkstal, K., Sterry, W., and Muche, J. M. (2002) A T-cell receptor γ polymerase chain reaction assay using capillary electrophoresis for the diagnosis of cutaneous T-cell lymphomas. *Diagn. Mol. Pathol.* **11,** 59–66.
6. Wood, G. S., Tung, R. M., Haeffner, A. C., Crooks, C. F., Liao, S., Orozco, R., et al. (1994) Detection of clonal T-cell receptor γ gene rearrangements in early Mycosis fungoides/Sezary syndrome by polymerase chain reaction and denaturing gradient gel electrophoresis (PCR/DGGE). *J. Invest. Dermatol.* **103,** 34–41.
7. Kneba, M., Bolz, I., Linke, B., Bertram, J., Rothaupt, D., and Hiddemann, W. (1994) Characterization of clone-specific rearranged T-cell receptor γ-chain genes in lymphomas and leukemias by the polymerase chain reaction and DNA sequencing. *Blood* **84,** 574–581.
8. Menke, M., Tiemann, M., Vogelsang, D., Boie, C., and Parwaresch, R. (1995) Temperature gradient gel electrophoresis for analysis of a polymerase chain reaction-based diagnostic clonality assay in the early stages of cutaneous T-cell lymphomas. *Electrophoresis* **16,** 733–738.
9. Scheller, U., Muche, J. M., Sterry, W., and Lukowsky, A. (1998) Detection of clonal T-cells in cutaneous T-cell lymphoma by polymerase chain reaction: comparison of Mutation Detection Enhancement® PAGE, temperature gradient gelelectrophoresis and fragment analysis on sequencing gels. *Electrophoresis* **19,** 653–658.
10. Lynas, C. and Howe, D. (1998) Simple, reliable detection of T cell clones by PCR-LIS-SSCP analysis of TCR rearrangement. *Mol. Cell. Probes* **12,** 41–48.
11. Wickham, C. L., Lynas, C., and Ellard, S. (2000) Detection of clonal T-cell populations by high resolutions PCR using fluorescently labelled nucleotides; evaluation using conventional LIS-SSCP. *J. Clin. Pathol. – Mol. Pathol.* **53,** 150–154.
12. Witzens, M., Möhler, T., Willhauck, M., Scheibenbogen, C., Lee, K. H., and Keilholz, U. (1997) Detection of clonally rearranged T-cell-receptor gamma chain genes from T-cell malignancies and acute inflammatory rheumatic diseases using PCR amplification, PAGE, and automated analysis. *Ann. Hematol.* **74,** 120–130.
13. Simon, M., Kind, P., Kaudewitz, P., Krokowski, M., Graf, A., Prinz, J., et al. (1998) Automated high-resolution polymerase chain reaction fragment analysis–a method for detecting T-cell receptor γ-chain gene rearrangements in lymphoproliferative diseases. *Am. J. Pathol.* **152,** 29–33.
14. Dippel, E., Assaf, C., Hummel, M., Schrag, H.-J., Stein, H., Goerdt, S., and Orfanos, C. E. (1999) Clonal T-cell receptor γ-chain gene rearrangement by PCR-based GeneScan analysis in advanced cutaneous T-cell lymphoma: a critical evaluation. *J. Pathol.* **188,** 146–154.

15. Ayling, J. F. and Iland, H. J. (1999) High-resolution analysis of gene rearrangements in lymphoid malignancies. *Pathology* **31,** 252–256.
16. Sprouse, J. T., Werling, R., Hanke, D., Lakey C., McDonnel, L., Wood, B. L., and Sabath, D. E. (2000) T-cell clonality determination using polymerase chain reaction (PCR) amplification of the T-cell receptor gamma-chain gene and capillary electrophoresis of fluorescently labeled PCR products. *Am. J. Clin. Pathol.* **113,** 838–850.
17. Beaubier, N. T., Hart, A. P., Bartolo, C., Willman, C. L., and Viswanatha, D. S. (2000) Comparison of capillary electrophoresis and polyacrylamide gel electrophoresis for the evaluation of T and B cell clonality by polymerase chain reaction. *Diagn. Mol. Pathol.* **9,** 122–131.
18. Ahnhudt, C., Muche, J. M., Dijkstal K., Sterry, W., and Lukowsky, A. (2001) An approach to the sensitivity of temperature gradient gel electrophoresis in the detection of clonally expanded T-cells in cutaneous T-cell lymphoma. *Electrophoresis* **22,** 33–38.
19. Lo, Y. M. D. (1998) Setting up a PCR laboratory, in *Clinical Applications of PCR* (Lo, Y. M. D., ed.), Humana, Totowa, NJ, pp. 11–17.
20. Serfling, U., Penneys, N. S., Zhu, W.-Y., Sisto, M., and Leonardi, C. L. (1993) Varicella-zoster virus DNA in granulomatous skin lesions following herpes zoster. *J. Cutan. Pathol.* **20,** 28–33.
21. TGGE Handbook (1993) Quiagen Inc., Chatsworth, CA, pp. 22–23.
22. Lee, S. C., Berg, K. D., Racke, F. K., Griffin, C. A., and Eshleman, J. R. (2000) Pseudo-spikes are common in histologically benign lymphoid tissue. *J. Mol. Diag.* **2,** 145–152.
23. Volkenandt, M., Soyer, H. P., Cerroni, L., Koch, O. M., Atzpodien, J., and Kerl, H. (1993) Molecular detection of clone-specific DNA in hypopigmented lesions of a patient with early evolving mycosis fungoides. *Br. J. Dermatol.* **128,** 423–428.
24. Trainor, K. J., Brisco, M. J., Wan, J. H., Neoh, S., Grist, S., and Morley, A. A. (1991) Gene rearrangement in B- and T-lymphoproliferative disease detected by the polymerase chain reaction. *Blood* **78,** 192–196.

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