Global Effects of BCR/ABL and TEL/PDGFRβ Expression on the Proteome and Phosphoproteome

IDENTIFICATION OF THE RHO PATHWAY AS A TARGET OF BCR/ABL*

Received for publication, September 15, 2004, and in revised form, November 2, 2004 Published, JBC Papers in Press, November 29, 2004, DOI 10.1074/jbc.M410598200

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Many leukemic oncogenes form as a consequence of gene fusions or mutation that result in the activation or overexpression of a tyrosine kinase. To identify commonalities and differences in the action of two such kinases, breakpoint cluster region (BCR)/ABL and TEL/PDGFRβ, two-dimensional gel electrophoresis was employed to characterize their effects on the proteome. While both oncogenes affected expression of specific proteins, few common effects were observed. A number of proteins whose expression is altered by BCR/ABL, including gelsolin and stathmin, are related to cytoskeletal function whereas no such changes were seen in TEL/PDGFRβ-transfected cells. Treatment of cells with the kinase inhibitor STI571 for 4-h reversed changes in expression of some of these cytoskeletal proteins. Correspondingly, BCR/ABL-transfected cells were less responsive to chemotactic and chemokinetic stimuli than non-transfected cells and TEL/PDGFRβ-transfected Ba/F3 cells. Decreased motile response was reversed by a 16-h treatment with STI571. A phosphoprotein-specific gel stain was used to identify TEL/PDGFRβ and BCR/ABL-mediated changes in the phosphoproteome. These included changes on Crkl, Ras-GAP-binding protein 1, and for BCR/ABL, cytoskeletal proteins such as tubulin, and Nedd5. Decreased phosphorylation of Rho-GTPase dissociation inhibitor (Rho GDI) was also observed in BCR/ABL-transfected cells. This results in the activation of the Rho pathway, and treatment of cells with Y27632, an inhibitor of Rho kinase, inhibited DNA synthesis in BCR/ABL-transfected Ba/F3 cells but not TEL/PDGFRβ-expressing cells. Expression of a dominant-negative RhoA inhibited both DNA synthesis and transwell migration, demonstrating the significance of this pathway in BCR/ABL-mediated transformation.

Chronic myeloid leukemia (CML) is a disease with a characteristic t(9:22) chromosomal translocation giving rise to the Philadelphia chromosome (1). This translocation results in the juxtaposition of the BCR (breakpoint cluster region) gene and the c-ABL oncogene resulting in the constitutive expression of a BCR/ABL fusion oncoprotein (2). Another chimeric leukemogenic oncogene product, TEL/PDGFRβ, isolated from chronic myelomonocytic leukemia (CMML) patients bearing a t(5,12) translocation, also has constitutive tyrosine kinase activity.

The transforming ability of BCR/ABL and TEL/PDGFRβ resides in their protein-tyrosine kinase activity. A number of signaling proteins activated by BCR/ABL have been identified including Ras, STAT5, protein kinase C, and phosphatidylinositol (PI) 3-kinase (3–6). BCR/ABL is also known to affect cell adhesion and motility, in part via its ability to bind actin (7), as well as altering the expression or activity of focal adhesion proteins (8). A detailed review of these pathways and downstream effectors can be found in Ref. 9. TEL/PDGFRβ can also activate signaling proteins such as PI 3-kinase, STAT1, STAT5, and stress-activated protein kinase (10–12). As with BCR/ABL, Myc is an essential element in transformation by TEL/PDGFRβ (13).

Recently, an inhibitor of the BCR/ABL protein-tyrosine kinase activity has shown great promise for the treatment of CML. STI571 (also known as Imatinib mesylate or GleevecTM) can inhibit BCR/ABL kinase activity both in vitro and in intact cells. STI571 can inhibit proliferation and induce apoptosis in BCR/ABL- and TEL/PDGFRβ-expressing cells (14, 15). Clinical trials using STI571 have demonstrated impressive hematologic and cytogenetic responses in CML patients (16) and also where rearrangement of the PDGFRβ gene has occurred (17). However, despite this success, there are cases of STI571-resistant BCR/ABL-positive CML (18). Thus the mechanistic detail of CML and CMML development remains a significant research objective to identify potential (common) targets for therapy.

Microarray experiments on primary CML samples have shown that the expression of BCR/ABL can alter transcript levels of genes involved in a wide variety of cellular processes, with poor disease prognosis in chronic phase being associated with changes in DNA repair, cell cycle, and cell adhesion path-

growth factor receptor; IL, interleukin; MGG, May-Grunwald-Giemsa; IPG, immobilized pH gradient; MALDI, matrix-assisted laser desorption/ionization; SDF-1, stromal cell-derived factor; LPA, lysophosphatic acid; GDI, GDP dissociation inhibitor; GAP, GTPase-activating protein; TTL, tubulin-tyrosine ligase; Rho GDI, Rho GDP dissociation inhibitor; PCNA, proliferating cell nuclear antigen; Arp, actin-related protein; STAT, signal transducer and activator of transcription; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; PBS, phosphate-buffered saline; MAP, mitogen-activated protein.

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Published, JBC Papers in Press, November 29, 2004, DOI 10.1074/jbc.M410598200

THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 280, No. 8, Issue of February 25, pp. 6316–6326, 2005

Printed in U.S.A.

This paper is available on line at http://www.jbc.org

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ways, as well as STAT5 and Myc pathway targets. Whether these are directly attributable to BCR/ABL activity or secondary changes within the tumor was not determined (19). Studies using transfected cells plus screening for altered transcript levels by subtractive hybridization show alterations in genes involved in, for example, the MAP kinase (TOPK, or T-LAK cell-­originated protein, a MAP kinase kinase), ubiquitination (HSPC150), or protein transport (NUP98 and RAN) pathways (20). A recent study using an inducible p210BCRABL system screened using cDNA microarrays revealed that BCR/ABL up-­regulates a range in interferon-­inducible genes, as well as transcription factors (STAT1, JUN), and cell growth and differentiation-­related genes (PCNA, REL, Stat3nun) (21). The situation with TEL/PDGFβ-­expressing cells remains relatively uninvestigated.

BCR/ABL can also initiate key changes that occur only at the proteome level. However, this phenomenon has also not been explored for TEL/PDGFβ. BCR/ABL is known to decrease expression of the Ab1 inhibitory proteins via a proteasome-­mediated mechanism (22). Other proteins whose expression alters with no apparent change in mRNA abundance as a consequence of BCR/ABL action include p53 (down-­regulated as a result of post-­translational-­mediated MDM2 overexpression) (23, 24) and CEBPs, whose mRNA is bound by an inhibitory poly(rC)-­binding protein hNRNP E2 (25). These proteins have important functions in hematopoietic cell survival and differentiation; thus, post-­translational regulation of protein levels can be seen to have a role in transformation processes.

A model system for comparing the transforming effects of oncogenes is the Ba/F3 murine cytokine-­dependent cell line transformed with BCR/ABL and TEL/PDGFβ, respectively (26). We have used two-­dimensional gel electrophoresis with the objective of comparing the effects of BCR/ABL and TEL/ PDGFβ on Ba/F3 cells by studying protein expression and phosphoprotein profiles with or without treatment with STI571. Very different outcomes are generated in Ba/F3 cells expressing these chimeric tyrosine kinases indicating pleiotropic mechanisms lead to or are associated with transformation by these 2 leukemogenic kinases.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—Ba/F3 cells were transfected with either an empty MSCV (murine stem cell virus) retroviral vector or MSCV containing the BCR/PDGFβ gene. Ba/F3-­MSCV or Ba/F3-­MSCV-­BCR/ABL or Ba/F3-­TEL/PDGFβ cells were maintained in suspension in culture in RPMI with 10% (v/v) fetal bovine serum. Ba/F3-­MSCV cells were grown in RPMI with 10% (v/v) fetal bovine serum supplemented with 1 mg/ml G418 (Sigma) and 1 mg/ml IL-­3 (R&D Systems, Minneapolis, MN). Prior to lysis, cells were either starved for 4 h in RPMI with 1% (v/v) bovine serum albumin (Sigma) and, where appropriate, 5 μM STI571, or were treated with 50 μM Y27632 (Merck Biosciences, Nottingham, UK) for 6 h. Cells were washed twice in ice-­cold PBS and once in ice-­cold 250 mM sucrose with 0.4 mM sodium orthovanadate (Sigma) and, cell pellets were stored at −80 °C until use. Further fractionation with Rho N19 was performed as described previously (27). Successfully transfected cells were selected for expression of green fluorescent protein marker using a flow cytometer (BD Biosciences). Cells were stained using May-­Grunwald-­Giemsa (MGG) stain as described previously (27).

**Two-­dimensional Gel Electrophoresis**—Cells were lysed in 9 M urea, 2 M thiourea, 4% (v/v) CHAPS, 1% (v/v) dithiothreitol, and 2% IPG buffer (Amersham Biosciences, Little Chalfont, UK), and protein concentration was determined using the Bio-­Rad modified Bradford protein assay. For silver-­stained gels, 100 μg of protein (1 mg for gels used for spot identification) was loaded by in-­gel rehydration onto 24 cm, pH 3–10 nl IPG strips (Amerham Biosciences) in a total volume of 450 μl of lysis buffer with a trace of Orange G (Sigma). For ProQ® Diamond (Molecular Probes, Leiden, The Netherlands)-­stained gels, 500 μg of protein were loaded onto 18-­cm pH 4–7 IPG strips (Amerham Biosciences) in a 350-μl final volume. Strips were rehydrated at room temperature overnight, transferred to a Multiphor II apparatus (Amersham Biosciences), and protein focused over 2 days for a total of 115 kV h. Second dimension separation was carried out on 10% SDS-­PAGE gels with 4% T stacking gel using a Hoeffer vertical electrophoresis system (Amerham Biosciences). 20 μg/mg gel overnight until the dye front reached the end of the gel. Detailed protocols can be found at www.lrf.umist.ac.uk.

**Silver Staining**—Analytical gels were stained using a silver staining kit from OWT separation systems (Portsmouth, NH) employing a modified protocol, as described in Ref. 28. Preparative grade gels were stained with the mass spectrometry-­compatible silver stain of Shevchenko et al. (29).

**ProQ® Diamond and Coomassie Blue Staining**—Gel staining with ProQ® Diamond was carried out according to the manufacturer’s instructions. Briefly, gels were fixed in 50% (v/v) methanol, 10% (v/v) trichloroacetic acid overnight, followed by a second fix for 1 h, washed in water 4×15 min, and then stained with ProQ® Diamond for 4 h in the dark. Gels were then destained with 50 mM sodium acetate at pH 4.0 with 4% (v/v) acetonitrile for 2 × 1 h, and a third wash overnight. Gels were imaged on a Typhoon™ 9600 scanner (Amerham Biosciences) using 532-­nm excitation and 610-­nm emission filters, with photomultiplier tube voltage set at 600 V. Selected gels were subsequently stained with Coomassie Blue to visualize the pattern of total protein and determine the specificity of the ProQ® Diamond stain. Gels were washed in water for 30 min, then stained in 10% (w/v) ammonium sulfate/2% (v/v) phosphoric acid with 0.1% Coomassie Blue G (Sigma) for 48 h. Staining solution was made up at least 24 h before use and diluted 4 parts stain to 1 part methanol before use. The gels were destained briefly in 50% (v/v) methanol and scanned on a Molecular Imager FX (Bio-­Rad). Gels were stained with ProQ® Diamond, imaged, and stored until analysis had been completed. Spots were then excised from these gels using as a template a 1:1 scale image of the gel. Following cutting, gels were rescanned to ensure that the correct spot had been excised.

**Image Analysis**—All gel analysis was performed using Progenesis (Non-­linear Dynamics, Newcastle, UK) software. Changes in spot intensity were deemed significant where the average normalized volume altered by greater than 1.5-­fold between samples, with p < 0.05 from a 2-­way Student’s t test on 3 or more replicate gels. Spot-­normalized volume defines the volume of a given spot as a percentage of the total volume of all spots in the gel.

**In-­gel Digestion and Protein Identification**—Spots excised from silver-­stained gels were first destained using a 50:50 mixture of 30 mM potassium III ferricyanide and 100 mM sodium thiocyanate. All spots were washed twice in water, then equilibrated three times with 25 mM ammonium bicarbonate, and dried by three washes with acetonitrile. Dried gel pieces were rehydrated in 25 μg/ml trypsin (Promega, Southampston, UK) in 25 mM ammonium bicarbonate on ice for 20 min. A further 20 μl of 25 mM ammonium bicarbonate was added to prevent drying out, and the gel was incubated at 37 °C overnight. Supernatant was then dried to around 2 μl in a SpeedVac centrifuge. Remaining gel pieces were resuspended in 2% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid and sonicated for 15 min. Extracted peptides were added to the concentrated supernatant and dried in a SpeedVac centrifuge. Peptides were reconstituted in 50% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid and spotted along with 0.5 μl of 10 mg/ml a-­cyano 4-­hydroxy cinnamic acid (CHCA, Sigma) in 50% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid onto a MALDI target for analysis using a reflector MALDI-­ToF mass spectrometer (M@LDI, Micromass, Manchester). Spectra were internally calibrated using trypsin autolysis fragments at m/z 842.509 or 2211.104. Tandem MS experiments were performed with an ABI QSTAR-­Pulsar XL mass spectrometer (ABI/Sciex, Thornhill, Ontario, Canada) using an on-­line liquid chromatography system (Dionex, Amsterdam, Netherlands). Dried peptides were reconstituted in 2% (v/v) acetonitrile, 0.1% (v/v) formic acid and were separated in a C18 reverse phase column on-­line with the Q-­STAR using a gradient of 2–60% acetone over 15 min. Protein identities were obtained by searching against either the SWISS-­PROT or NCBI non-­redundant data bases using Mascot (Matrix Science, London).

**Western Blotting**—To confirm changes in the expression of cytoskeletal proteins and cytoskeleton-­associated proteins, 20 μg of cell lysate was loaded on a 10% one-­dimensional SDS-­PAGE gel (this was increased to 50 μg of lysate on a 15% SDS-­PAGE gel for stathmin detection), and transferred to a nitrocellulose membrane using an Electro-­Blot apparatus (Web Scientific, Crewe, UK) in 192 μM glycine, 25 mM Tris, 20% (v/v) methanol at 100 V for 45 min. Membranes were blocked in PBS with 0.1% Tween-­20 (PBS-­T) with 5% (w/v) dried nonfat milk (Marvel) at 4 °C overnight. The membrane was then washed once in PBS-­T, and incu...
bated with primary antibodies for 1 h as follows; anti-stathmin rabbit polyclonal antibody (30) (a gift from E. Sobel, INSERM, Paris) at 1:10,000, rabbit anti-Arp2 (Chemicon International, Chandlers ford, UK) at 1:1,000, and rabbit anti-gelsolin serum (31) (a gift from D. Kwiatkowski, Harvard Medical School, Boston, MA) at 1:10,000. Membranes were washed three times, incubated in peroxidase-conjugated mouse anti-rabbit Ig (Amersham Biosciences) at 1:10,000 for 1 h, and washed four times. All antibody dilutions were in PBS-T with 1% w/v milk. All washes were performed with PBS-T. All antibody dilutions were in PBS-T with 1% w/v milk. All washes were performed with PBS-T. Detection was carried out using Supersignal West Pico chemiluminescent substrate (Pierce), and signal detected on Kodak x-ray (Sigma) film. All blots were subsequently stained with Coomassie Blue to ensure equal loading.

Chemotaxis and Cell Motility Assay—The migration of Ba/F3 cells in response to agonists was assessed using a 24-well transwell plate (Costar, Corning, New York). These consisted of two wells separated by a membrane containing 5-micron pores. Cells (1–2 × 10⁵ in 100 µl) were placed in the top well, and agonists were added to the top and/or bottom wells (bottom well volume 600 µl) in Fishers medium plus 20% (v/v) batch-tested horse serum. After 6–8 h of incubation at 37 °C in a 5% CO₂ humidified incubator, viable cells in the lower well were counted using trypan blue (Sigma). In no experiment was cell viability less than 98% in either top or bottom well after incubation.

[^H]Thymidine Incorporation Assay for Cell Proliferation—[^H]Thymidine incorporation assays were performed as described previously (32).

**RESULTS**

**BCR/ABL- and TEL/PDGFRβ-transfected Ba/F3 Cells Display Different Changes in Protein Expression That Are Partially Reversed by STI571**—Ba/F3 cells expressing either BCR/ABL or TEL/PDGFRβ exhibit a number of behavioral changes. Most notable among these, is that transduced cells no longer require the presence of growth factor (IL-3) for their survival or proliferation (12, 26). Protein from Ba/F3 cells transfected with an empty vector (MSCV), and cells transfected to express either BCR/ABL or TEL/PDGFRβ, (with or without treatment with STI571 for 4 h) were separated using a pH 3–10 immobilized
Proteome/Phosphoproteome Effects of BCR/ABL and TEL/PDGFRβ

Identities of proteins whose expression is altered by expression of BCR/ABL or TEL/PDGFRβ, and/or treatment with STI571

Proteins whose expression level changes with BCR/ABL or TEL/PDGFRβ with a significance greater than 0.05 and a fold change greater than 1.5 times are shown below. M and pI given are those predicted from amino acid sequence. In all cases the molecular mass and pI correlated to the region of the gel where the protein was found, confirming identity. Mean normalized spot volume is the average value from five replicate gels for each condition.

| Spot | Identity | M (kDa) | pI | SWISS-Prot/NCBI | Mean normalized spot volume ± S.E. |
|------|----------|---------|----|----------------|-----------------------------------|
| Ba/F3 | Oncogene | +STI571 |

**TABLE I**

| Proteins decreased by BCR/ABL expression | kDa | Identity | M | pI | SWISS-Prot/NCBI |
|------------------------------------------|-----|----------|----|----|----------------|
| CB1 | GRP78 | 70.5 | 5.01 | P20029 | 0.271 ± 0.009 |
| CB2 | Gelsolin | 83.3 | 5.72 | P13120 | 0.120 ± 0.008 |
| CB3 | Adseverin (Siderin) (Gelsolin-like protein) | 80.3 | 5.64 | Q66064 | 0.072 ± 0.009 |
| CB4 | Adseverin (Siderin) (Gelsolin-like protein) | 80.3 | 5.64 | Q66064 | 0.151 ± 0.013 |
| CB5 | Thimet oligopeptidase 1 | 78.0 | 5.72 | Q8HD24 | 0.113 ± 0.016 |
| CB6 | Not identified | — | — | — | 0.145 ± 0.021 |
| CB7 | Unknown protein— similar to Serpin E1 | 42.5 | 5.85 | GI1384881 | 0.219 ± 0.003 |
| CB8 | Apol | 44.7 | 6.30 | O15142 | 0.249 ± 0.037 |
| CB9 | Eukaryotic translation initiation factor 3, subunit 3 | 39.8 | 6.19 | Q9112K | 0.101 ± 0.008 |
| CB10 | Transaldolase | 37.4 | 6.57 | Q63092 | 0.128 ± 0.003 |
| CB11 | Unknown protein— similar to HSCARG | 34.4 | 6.37 | GI24431937 | 0.070 ± 0.013 |
| CB12 | Proteasome activator complex subunit 1 | 28.7 | 5.73 | P97371 | 0.111 ± 0.018 |
| CB13 | Proteasome subunit - beta type 3 | 23.0 | 6.15 | Q8R4P1 | 0.112 ± 0.014 |
| CB14 | Phosphatidylinositol-binding protein | 20.7 | 5.19 | P70296 | 0.342 ± 0.029 |

| Proteins increased by BCR/ABL expression | kDa | Identity | M | pI | SWISS-Prot/NCBI |
|------------------------------------------|-----|----------|----|----|----------------|
| B3 | Protein kinase C, δ | 77.5 | 7.20 | P28867 | 0.047 ± 0.004 |
| B4 | VEGF-C precursor | 73.0 | 7.89 | P97953 | 0.000 ± 0.000 |
| B5 | Not identified | — | — | — | 0.013 ± 0.005 |
| B6 | Not identified | — | — | — | 0.013 ± 0.001 |
| B7 | Tropomyosin a3, isoform 2 | 32.8 | 4.68 | P21107 | 0.140 ± 0.019 |
| B8 | Tropomyosin a3, isoform 2 | 32.8 | 4.68 | P21107 | 0.140 ± 0.019 |
| B9 | Tropomyosin a3, isoform 2 | 32.8 | 4.68 | P21107 | 0.140 ± 0.019 |
| B10 | Not identified | — | — | — | 0.153 ± 0.001 |
| B11 | Not identified | — | — | — | 0.153 ± 0.001 |
| B12 | 14-3-3 protein epsilon (mouse) | 29.1 | 4.63 | P42655 | 0.040 ± 0.002 |
| B13 | 14-3-3 protein epsilon (mouse) | 29.1 | 4.63 | P42655 | 0.040 ± 0.002 |
| B14 | 14-3-3 protein zeta/delta (mouse) | 27.8 | 4.73 | P35215 | 0.040 ± 0.002 |
| B15 | 14-3-3 protein zeta/delta (mouse) | 27.8 | 4.73 | P35215 | 0.040 ± 0.002 |
| B16 | Growth factor receptor-bound protein 2, GBR2 | 25.2 | 5.72 | Q66031 | 0.025 ± 0.000 |
| B17 | Antioxidant protein 2 and isopentenyl-diphosphate β-isomerase 1 | 24.7263 | 5.72/5.79 | O00709/P58044 | 0.026 ± 0.011 |
| B18 | Putative lysosomal protein | 11.1 | 8.55 | Q15685 | 0.000 ± 0.000 |
| B19 | Stathmin (neuroprotein 18) | 17.1 | 5.76 | P54227 | 0.002 ± 0.002 |
| B20 | Stathmin (neuroprotein 18) | 17.1 | 5.76 | P54227 | 0.002 ± 0.002 |
| B21 | PCNA | 28.8 | 4.66 | P17918 | 0.210 ± 0.071 |

| Proteins increased by STI571 in BCR/ABL-expressing cells | kDa | Identity | M | pI | SWISS-Prot/NCBI |
|------------------------------------------|-----|----------|----|----|----------------|
| B3 | Single strand selective monofunctional uracil DNA glycosylase | 30.7 | 6.40 | Q60631 | 0.145 ± 0.003 |

| Proteins decreased by TEL/PDGFRβ expression | kDa | Identity | M | pI | SWISS-Prot/NCBI |
|------------------------------------------|-----|----------|----|----|----------------|
| CT1 | GRP78 | 70.5 | 5.01 | P20029 | 0.271 ± 0.009 |
| CT2 | 5/3- Nucleotidase, cytosolic | 23.0 | 5.31 | Q8JM14 | 0.029 ± 0.007 |
| CT3 | Phosphatidylinositol-binding protein | 20.7 | 5.19 | P70296 | 0.392 ± 0.021 |

| Proteins increased by TEL/PDGFRβ expression | kDa | Identity | M | pI | SWISS-Prot/NCBI |
|------------------------------------------|-----|----------|----|----|----------------|
| T1 | Follistatin-related protein 1 | 32.5 | 5.41 | Q62536 | 0.021 ± 0.010 |
| T2 | Follistatin-related protein 1 | 32.5 | 5.41 | Q62536 | 0.021 ± 0.010 |
| T3 | Tropomyosin a3, isoform 2 | 32.8 | 4.68 | P21107 | 0.140 ± 0.019 |
| T4 | Not identified | — | — | — | 0.109 ± 0.041 |
| T5 | 14-3-3 protein zeta/delta | 27.8 | 4.73 | P35215 | 0.040 ± 0.002 |
| T6 | 14-3-3 protein zeta/delta | 27.8 | 4.73 | P35215 | 0.040 ± 0.002 |
| T7 | NADH ubiquinone oxidoreductase 24-kDa subunit | 23.8 | 5.31 | Q6BD3E | 0.001 ± 0.000 |
| T8 | Not identified | — | — | — | 0.067 ± 0.027 |
| T9 | PCNA | 28.8 | 4.66 | P17918 | 0.210 ± 0.071 |
| T10 | Not identified | — | — | — | 0.258 ± 0.024 |

pH gradient gel in the first dimension followed by SDS-PAGE. The spot patterns (from three gels) were analyzed to detect significant differences in levels of expression of specific proteins between the control Ba/F3 cells, Ba/F3-BCR/ABL cells and TEL/PDGFRβ-transfected Ba/F3 cells (Fig. 1). The latter two samples were also treated with STI571 and then compared with non-treated controls using two-dimensional electrophoresis (Fig. 1). The major changes in protein levels observed were quantified using Progenesis software. Proteins shown to alter in their expression according to the gel patterns were identified where possible using mass spectrometry, as shown in Table I. Ba/F3-BCR/ABL cells showed significant changes in 32 proteins compared with Ba/F3 cells. Several protein functional groups can be identified in the list of proteins that change as a consequence of BCR/ABL expression, for example signaling proteins such as 14-3-3 proteins and Grb2, metabolic enzymes, and cytoskeleton regulators (see Table I). TEL/PDGFRβ induced 13 significant changes in protein expression, and these
showed some overlap to those seen with BCR/ABL. However there are a significantly fewer changes induced by TEL/PDGFRβ than by BCR/ABL: each tyrosine kinase, while inducing growth factor independence is not affecting the proteome in a similar fashion.

**BCR/ABL but Not TEL/PDGFRβ Induces Changes in Cytoskeletal Proteins and Regulators of the Motile Response**—When compared with control Ba/F3 cells, changes in cytoskeletal-associated proteins such as decreased levels of gelsolin, adseverin, and actin-related protein 2 (Arp2), and increased expression of stathmin are seen in BCR/ABL expressing Ba/F3 cells but not TEL/PDGFRβ-transfected cells. Gelsolin and adseverin are closely related proteins that play a role in actin filament severing and capping, and are therefore closely involved in cell motility (33). Arp2 is part of the Arp2/3 complex that is also associated with formation of short, branched actin filaments in the leading edge of motile cells (34). In contrast, tropomyosin, several forms of which were increased by both BCR/ABL and TEL/PDGFRβ in this study, has been shown to cause annealing of gelsolin capped actin filaments (35), and can inhibit actin branch formation and nucleation by the Arp2/3 complex (36). TEL/PDGFRβ-transfected cells carry only one of these changes in proteins known to regulate motility and morphology, namely tropomyosin. This has been confirmed by Western blotting for gelsolin, actin-related protein, and stathmin (Fig. 2, A–C).

A notable change seen in both TEL/PDGFRβ- and BCR/ABL-transfected Ba/F3 cells compared with control Ba/F3 cells is proliferating cell nuclear antigen (PCNA). As all three populations are actively cycling (the latter via the inclusion of IL-3 to cultured Ba/F3 cells) this is a significant oncogene-induced difference in the proteome. Again, this finding was confirmed by Western blot analysis (Fig. 2D). 14-3-3 proteins are also altered, both in TEL/PDGFRβ- and BCR/ABL-transfected cells.

The treatment of Ba/F3-BCR/ABL cells with STI571 resulted in alterations in the two-dimensional-PAGE pattern, with the increased expression of 5 proteins (including adseverin) and the decrease of seven proteins following drug treatment (see Table I). Cells were treated with STI571 for only 4 h (as the cells began to undergo drug-induced apoptosis after this time). The full extent of the changes in the specific proteins listed above in viable cells may not therefore be represented in this 4-h incubation. Nonetheless the involvement of a caspase-mediated cleavage of gelsolin in apoptosis (37) gives a clear reason for avoiding examination of the proteome of STI571-stimulated apoptosis in Ba/F3 cells. In fact we view it as indicative of the success of this strategy that gelsolin levels did not fall as a consequence of the 4-h incubation with STI571. In the case of Ba/F3-TEL/PDGFRβ cells no spots were increased in expression levels by STI571 while seven spots were decreased. Thus STI571 can reverse some of the effects of TEL/PDGFRβ- or BCR/ABL transduction after just 4 h. Since STI571 has no effect on untransduced Ba/F3 cells (see Ref. 38 and phosphoprotein analysis described in this report), this combination was not analyzed.

**BCR/ABL but Not TEL/PDGFRβ-expressing Ba/F3 Cells Display Altered Response to Chemotactic Stimuli**—The weight of changes driven by BCR/ABL associated with the cytoskeleton and its regulation and the paucity of such effects in TEL/PDGFRβ-transfected cells led us to consider the response of these cell lines to chemotactic factors such as stromal-derived factor 1 (SDF 1).

Importantly, oncogene-transduced cells show no changes in cell morphology as compared with mock-transfected cells (Fig. 3A). We assessed the response of Ba/F3 control, Ba/F3-TEL/PDGFRβ, and Ba/F3-BCR/ABL cells to the chemokine SDF-1 (which induces a chemotactic response) in the presence and absence of the stem cell chemokinetic factor, lyposphatidic acid (LPA). LPA has been shown to potentiate motile responses via the Vav guanyl nucleotide exchange factor that is activated by BCR/ABL (39). BCR/ABL expression decreased the response to both SDF-1 and LPA, and these effects were not relieved by addition of STI571 during the assay (Fig. 3B), although prior treatment with STI571 did abrogate this partial loss of motile function (Fig. 3B). The incubation with STI571 had no effect on cell viability. The lack of ability of the BCR/ABL kinase inhibitor to reverse the decreased motile response of the Ba/F3 cells correlates with the inability of STI571 to affect a change in the levels of all the cytoskeletal proteins whose levels are modulated by BCR/ABL over a short incubation time. We have previously shown that decreased gelsolin and adseverin expression is associated with a low level motile response in hematopoietic stem cells (40). TEL/PDGFRβ showed no alteration in motility, correlating with the findings from the proteomics screen where changes in cytoskeletal proteins, with the exception of tropomyosin, were found only in BCR/ABL-expressing cells. Altered expression patterns of these proteins can therefore provide an explanation for the observed decrease in motility in the chemotaxis assays displayed by the BCR/ABL-expressing cells.

**Identification of Alterations in Phosphoprotein Levels Using Two-dimensional Electrophoresis and Phosphoprotein Staining**—As a rapid and objective means of identifying major phosphorylation events governed by BCR/ABL and TEL/PDGFRβ we carried out an analysis of two-dimensional gels from control
and transfected cells using a phosphoprotein stain, ProQ® Diamond. Triplicate gels containing lysate from Ba/F3-MSCV (empty vector) Ba/F3-TEL/PDGFRβ, and Ba/F3-BCR/ABL cells with or without treatment with STI571 were produced with a pH 4–7 gradient in the first dimension. Preliminary experiments have shown that the majority of phosphoproteins are resolved on this gradient. Furthermore the majority of changes in the silver staining experiments described above were in this region and using a narrower pH range allowed more protein to be loaded, improving the probability of detecting lower abundance phosphoproteins. The use of known quantities of enriched phosphoproteins confirmed that there is an ability to detect the phosphoproteins casein and ovalbumin at the low micromole level after gel electrophoresis, with little or no detection of non-phosphorylated proteins such as albumin or alcohol dehydrogenase (data not shown).

When 500 µg of protein from Ba/F3 cells were loaded in two-dimensional electrophoresis experiments and stained for phosphoproteins, an average of 296 ± 7 spots were observed on the pH 4–7 gels for Ba/F3 cells, 290 ± 17 for Ba/F3-BCR/ABL cells, and 308 ± 14 for Ba/F3-TEL/PDGFRβ cells (Fig. 4). The addition of STI571 reduced the number of spots in BCR/ABL cells by 23.1% and in TEL/PDGFRβ cells by 11%. This approach detects changes in major phosphoproteins and as such the similarities in numbers of stained proteins is to be anticipated, as 30% of the proteome is predicted to be made up of phosphoproteins and we observe ~1000 proteins from these samples with a total protein stain (silver). To detect differences between the molecular mode of action of BCR/ABL and TEL/PDGFRβ analysis of these spot patterns was followed by mass spectrometry where sufficient protein was available to enable detection of the protein in question (Table III). No significant differences were identified between the control Ba/F3 cells in the presence or absence of STI571, supporting the observation that STI571 treatment has no effect on untransfected Ba/F3 cells (38). BCR/ABL-transfected Ba/F3 cells displayed significant differences in phosphoprotein-specific staining as compared with control cells and TEL/PDGFRβ-transfected cells.

Expression of BCR/ABL affected the phosphoprotein staining of 32 spots. It caused a significant increase (greater than 2-fold, p < 0.05) in the fluorescence of 14 protein spots, and this effect is reversed with 4 h of STI571 treatment in 5 cases. BCR/ABL also causes decreased staining (greater than 2-fold decrease in signal intensity, p < 0.05) of 18 protein spots compared with Ba/F3 cells, with 7 of these changes being reversed affected by STI571. Interestingly, 1 spot appeared to alter upon STI571 treatment although it was not significantly different following BCR/ABL expression. This spot was found to contain a Rab GDP dissociation inhibitor β2, which is increased by BCR/ABL in a distinct (adjacent) spot implying an increase in a phosphorylated form in BCR/ABL expressing cells that is reversed upon treatment with STI571. The fact that only one form increases phosphorylation in BCR/ABL yet both are decreased by STI571 possibly suggests that addition of STI571 has a secondary effect on Rab GDI β2, which cannot be determined using the current experimental design.

Where the relative intensities of the silver- or Coomassie Blue-stained and ProQ® Diamond stained spots could be compared, the data implied that the majority of changes detected by this staining method are caused by alterations in specific phosphorylation per se. Only 60% of the changes could confidently be assessed in this manner due to the differences in spot patterns produced by the two stains. Likewise, the majority of proteins identified as being altered in expression in the silver staining experiments (Fig. 1) do not show any altered phosphoprotein staining.

The most notable differences seen in BCR/ABL Ba/F3 cells include Crkl and enolase, both well known downstream targets of BCR/ABL. Each protein spot increased in ProQ® Diamond staining in BCR/ABL expressing cells compared with control cells (Fig. 4 and Table II). This validates the method employed. Noteworthy is the fact that BCR/ABL decreased phosphorylation in a large number of proteins as well as increasing phosphorylation in others. Proteins whose fluorescence staining was modulated included those involved in protein synthesis (eukaryotic initiation factor 4A-1), chaperone proteins (Hsp90), proteasomal proteins (sumo 1-activating enzyme subunit 2, 26 S protease regulatory subunit), cytoskeletal proteins (tubulin β5, tubulin α6), and signal transduction proteins, including Toll-like receptor 2, Ras-GTPase-activating protein-binding protein, and Rho GDI. The only proteins commonly affected by TEL/PDGFRβ and BCR/ABL were Ras-GTPase-activating protein-binding protein and eukaryotic initiation factor 4A-1. On three occasions two spots, one identified as being increased by oncogene and the other decreased, were found to contain the same protein (Rab GDP dissociation inhibitor β2 in the BCR/ABL analysis, Ras GAP-binding protein, and tubulin α-6 in the TEL/PDGFRβ analysis). Presumably these spots contained the different phosphorylated forms of these proteins.

The dephosphorylation of Rho GDI1 in Ba/F3-expressing cells is of interest, as the Rho GTPase is known to be constitu-
tively activated in BCR/ABL-expressing Ba/F3 cells (41). We confirmed the increase in GTP-bound Rho in BCR/ABL Ba/F3 cells (data not shown). As a principal target of Rho is Rho kinase we have analyzed the effect of Rho kinase inhibition on BCR/ABL-transfected Ba/F3 cells using the Y27632 inhibitor. The Rho Kinase Inhibitor Y27632 Inhibits BCR/ABL-mediated Proliferation—Rho kinase is involved in motile behavior in many cell types (42). As expected, the Rho kinase inhibitor Y27632 markedly decreased the motile response of control, BCR/ABL- and TEL/PDGFRα/H9252-transfected Ba/F3 cells in transwell migration assays (data not shown) demonstrating the role of the kinase in agonist-stimulated motile responses in these cells (43).

The potential contribution of Rho kinase to the BCR/ABL- and TEL/PDGFRα-mediated mitogenic response was also analyzed. TEL/PDGFRα-stimulated DNA synthesis was inhibited by 15 ± 2% (mean ± S.E., n = 5) by 50 μM Y27632, consistent with the role of Rho in cell cycle progression. BCR/ABL-mediated effects on DNA synthesis were inhibited more markedly, by 35 ± 11% by addition of 50 μM Y27632 (Fig. 5). Decreased inhibition was seen in the presence of IL-3. No additive or synergistic effects of the BCR/ABL inhibitor STI571 and the Rho kinase inhibitor Y27632 were observed at either suboptimal or optimal doses. This suggests that inhibition of BCR/ABL is also leading to Rho kinase inhibition, because no further effects on the Rho pathway can be observed upon addition of a Rho inhibitor when STI571 is present. This is consistent with data reported by Harnois et al. (41). Thus Rho kinase represents a novel target downstream from BCR/ABL.

Dominant-negative Rho Decreases BCR/ABL-mediated Proliferative Responses—To confirm the role of Rho in BCR/ABL-mediated proliferative responses we transduced Ba/F3 cells and Ba/F3-BCR/ABL cells with dominant-negative Rho (RhoAN19). The Ba/F3-BCR/ABL-N19 cells exhibited a 69 ± 3% decrease in the rate of [³²H]thymidine incorporation (mean ±

![Fig. 4. ProQ Diamond-stained two-dimensional gels (pH 4–7) containing 500 μg of total protein lysate.](image)
### Table II

| Spot | Identity | M | pL | SWISS-Prot/NCBI | Mean normalized spot volume ± S.E. |
|------|----------|---|----|-----------------|-----------------------------------|
|      |          |   |    |                 | Ba/F3                              |
|      |          |   |    |                 | Oncogene                          |
|      |          |   |    |                 | +STI571                           |

| Signal decreased by BCR/ABL expression | kDa | Signal increased by BCR/ABL expression |
|----------------------------------------|-----|--------------------------------------|
| BM1 Heat shock protein HSP 90-1 (HSP 90) | 63.1| 4.97 | P11499 | 4.25 ± 0.32 | 0.000 ± 0.000 | 0.000 ± 0.000 |
| BM2 Ubiquitin-like 1 (Sentrin)-activating enzyme E1B, a.k.a. SUMOL-activating enzyme subunit | 70.5| 5.09 | Q6Z1P9 | 0.038 ± 0.038 | 0.126 ± 0.025 | 0.039 ± 0.001 |
| BM4 Unknown protein | 74.0| 5.39 | Q7TPC3 | 0.139 ± 0.021 | 0.017 ± 0.007 | 0.017 ± 0.003 |
| BM5 Histone deacetylase 1 | 55.0| 5.31 | O96175 | 0.649 ± 0.060 | 0.238 ± 0.027 | 0.261 ± 0.037 |
| BM6 Ras-GTPase-activating protein-binding protein 1 | 51.8| 5.41 | P97855 | 0.551 ± 0.067 | 0.099 ± 0.044 | 0.195 ± 0.013 |
| BM7 Ras-GTPase-activating protein-binding protein 1 | 51.8| 5.41 | P97855 | 0.707 ± 0.042 | 0.204 ± 0.058 | 0.119 ± 0.019 |
| BM8 60-kDa heat shock protein | 60.9| 5.1 | P19226 | 0.162 ± 0.049 | 0.000 ± 0.000 | 0.000 ± 0.000 |
| BM9 26 S protease regulatory subunit 6B and HSC70-interacting protein | 47.3| 4.4 | P54775/Q9894L7 | 0.749 ± 0.086 | 0.217 ± 0.029 | 0.000 ± 0.000 |
| BM10 Eukaryotic initiation factor 4A-I | 46.1| 5.32 | P04765 | 0.212 ± 0.046 | 0.026 ± 0.016 | 0.063 ± 0.012 |
| BM11 Septin 2 (NEDD5 protein) | 41.4| 6.10 | P42208 | 0.437 ± 0.140 | 0.089 ± 0.041 | 0.094 ± 0.002 |
| BM12 Elongation factor 16 | 31.3| 4.93 | P19776 | 0.350 ± 0.163 | 0.000 ± 0.000 | 0.623 ± 0.054 |
| BM13 Rho GDP-dissociation inhibitor 1 (Rho-GD11) | 23.4| 5.12 | Q69PT1 | 0.373 ± 0.026 | 0.135 ± 0.003 | 0.136 ± 0.005 |
| SB1 Heat shock cognate 71-kDa protein | 70.8| 5.37 | P08109 | 0.074 ± 0.022 | 0.034 ± 0.004 | 0.097 ± 0.007 |
| SB2 Tubulin β5 or β2 | 49.6| 4.78 | P05218 | 0.632 ± 0.057 | 0.179 ± 0.038 | 0.294 ± 0.021 |
| SB3 Unknown protein | 46.1| 6.52 | Q191Y1 | 0.120 ± 0.027 | 0.064 ± 0.014 | 0.161 ± 0.004 |
| SB4 Cytoplasmic nonspecific dipeptidase | 52.7| 5.43 | Q96FA2 | 0.266 ± 0.056 | 0.047 ± 0.001 | 0.312 ± 0.075 |
| SB7 Splicing factor, arginine/serine-rich 9 | 25.6| 8.24 | gi| 15385016 | 0.100 ± 0.028 | 0.044 ± 0.017 | 0.115 ± 0.022 |
| BS5 Rab-GDP dissociation inhibitor β-2 | 50.5| 5.93 | Q61598 | 0.343 ± 0.033 | 0.075 ± 0.048 | 0.000 ± 0.000 |

Signal decreased by BCR/ABL expression:

| Signal increased by BCR/ABL expression |
|--------------------------------------|

**Note:** BM9 is absent in Ba/F3 cells, and is present as an unresolved train in the oncogene-transfected cells, making it impossible to get accurate quantification although it does represent a significant change.

S.E., n = 4) compared with Ba/F3-BCR/ABL cells. In the presence of IL-3, the difference between the Ba/F3-BCR/ABL-N19 and the Ba/F3-BCR/ABL cells was reduced to 26 ± 3%. The effect of RhoA N19 on control Ba/F3 cells was a 34 ± 7% decrease in IL-3-stimulated rate of DNA synthesis. Thus the BCR/ABL-mediated events leading to proliferation are more dependent on the Rho pathway.

Rhoa N19 also decreased the rate of SDF-1-stimulated transwell migration in Ba/F3 cells by 36 ± 8% (mean ± S.E., n = 3) and in Ba/F3-BCR/ABL cells by 60 ± 12%. The chemotactic response is significantly affected by inhibition of Rho either in the presence or absence of BCR/ABL activity.

**Discussion**

Many aspects of BCR/ABL-mediated signal transduction events have now been elucidated. In the case of the TEL/
Both TEL/PDGFRβ mediated events in hematopoietic cells far less is known than for BCR/ABL. The initial objective of the work described here was to compare and contrast downstream effects of these 2 oncogenes on the proteome and major effects observed on the phosphoproteome. We have shown that, while both TEL/PDGFRβ and BCR/ABL can induce growth factor independence in Ba/F3 cells, the effects on the major elements of the proteome and the phosphoproteome are very different. Some of the BCR/ABL- and TEL/PDGFRβ-mediated effects on the proteome we observed have been seen previously in leukemia cells. For example, the Grb2 signaling protein, found to be up-regulated by BCR/ABL expression, is known to be a key component of BCR/ABL signaling (3), and is essential for the growth of BCR/ABL-expressing cells and the development of CML (44, 45). Likewise, stathmin (oncoprotein 18), a microtubule-destabilizing protein, has been previously shown to be up-regulated in ALL and AML samples, which can both contain a BCR/ABL fusion protein (46, 47). Stathmin is a key regulator in proliferation, with phosphorylation (occurring in response to cell proliferation) reducing its binding to tubulin and promoting the formation of microtubules key in cell division.

Another protein that has been linked to BCR/ABL signaling and was shown here to be regulated by BCR/ABL and TEL/PDGFRβ is the 14-3-3 family of proteins. 14-3-3 family members, tropomyosin, phosphatidylethanolamine-binding protein, and actin-related protein 2, and other actin-skeletal-associated proteins. BCR/ABL induced decreased levels of gelsolin, adseverin, and actin-related protein 2, and increased expression of tropomyosin, whereas of these only tropomyosin was affected by TEL/PDGFRβ. This observation is important, as altered adhesive and motile properties of CML cells has been linked to their escape from the marrow and loss of regulation by the marrow microenvironment (53).

Gelsolin and adseverin play a key role in cell motility (54) via actin filament severing and end capping (33). Their activity can be modulated by tropomyosin, which anneals gelsolin-capped actin and forms long actin filaments (35). The expression of adseverin in a megakaryoblastic leukemia model induces differentiation and inhibits proliferation and transforming ability (55). Arp2 also has a role in the formation of short, branched actin filaments in motile cells (34). Arp2 is part of the Arp2/3 complex that is key in the formation of new actin branches (56). Interestingly, tropomyosin can also impact on this process by inhibiting actin branch formation and nucleation by the Arp2/3 complex (36).

To confirm that BCR/ABL did indeed have an effect on motility that was not seen in TEL/PDGFRβ, transwell migration assays were performed to assess the response to the chemokine SDF-1 and LPA. BCR/ABL-expressing cells displayed a lower rate of motility than both control and TEL/PDGFRβ-expressing cells, and this effect could be reversed by the addition of STI571 to the cells. This confirms that not only does BCR/ABL affect motile behavior (see Ref. 43), but also that TEL/PDGFRβ does not, providing further proof that the two oncogenes affect different pathways.

By comparison to BCR/ABL, TEL/PDGFRβ had little effect on the proteome, and is certainly not affecting the panoply of cytoskeletal-associated proteins potentiated by BCR/ABL expression. This correlates with the lack of effect of TEL/PDGFRβ on motile responses to chemokines (see Fig. 3). The only proteins identified whose expression is altered by both oncogenes are the 14-3-3 family members, tropomyosin, phosphatidylethanolamine-binding protein, and PCNA, a component of the DNA polymerase (57). The presence of both the 14-3-3 proteins and phosphatidylethanolamine-binding protein in both lists may indicate that both oncogenes have an effect on Raf.

A potentially important protein affected by TEL/PDGFRβ that did not appear to change when BCR/ABL is expressed is follistatin-related protein 1. This TGF-β-inducible protein (58), which can also be up-regulated upon protein kinase C activation (59), has been previously demonstrated to down-regulate the activity of activin A (60), a TGF-β superfamily member. Activin A has been shown to have an important role in hematopoiesis, and can inhibit colony formation by peripheral blood granulocyte-monocyte colony-forming units when stimulated with IL-3. Activin A also abolishes the increased DNA synthesis seen when these cells are treated with IL-3 (61), suggesting
that it plays a role in the maintenance of differentiation. Inhibition of this protein, for example by up-regulation of follistatin-related protein, may be important in leukemogenesis (62).

Whereas TEL/PDGFRβ is not affecting motility, it most certainly affects growth of Ba/F3 cells. To provide information about the relative effects of BCR/ABL and TEL/PDGFRβ on processes that may govern proliferation, we utilized the ProQ® Diamond phosphoprotein-specific gel stain (63, 64). Our objective was to identify major phosphorylation events associated with expression of the BCR/ABL and TEL/PDGFRβ, respectively.

The phosphoprotein alterations observed between the control and BCR/ABL-transfected samples again include a number of proteins, which are already known to be targets of the ABL kinase. Crkl, known to be one of the major proteins that binds to and is phosphorylated by BCR/ABL (65–68). Heat shock protein 90 (Hsp90), is apparently highly phosphorylated in control cells but this is not so in BCR/ABL-expressing cells. Hsp90 has been identified as a potential therapeutic target in CML. It binds to BCR/ABL in a complex with p23 (69) and protects the BCR/ABL protein from proteasome-mediated degradation. Cells containing mutated BCR/ABL, resistant to STI571 treatment, are still responsive to Hsp90 inhibitors and show increased rates of BCR/ABL degradation (70). Phosphorylation of Hsp90 appears to release the Hsp90 substrate binding (71), and so the dephosphorylation seen in these cells is probably key to the stabilization of the BCR/ABL protein. The above proteins are unaffected by TEL/PDGFRβ expression.

The Ras-GTPase-activating protein-binding protein 1 was dephosphorylated by expression of BCR/ABL and TEL/PDGFRβ. It has been shown that this protein is hyperphosphorylated in quiescent cells (72). A key point here is that the Ba/F3 control cell population had been maintained in an actively proliferating state by maintaining the cells in IL-3 thus the difference between BCR/ABL-transfected and mock-transfected Ba/F3 will consist of more than the difference between BCR/ABL-transfected and mock-transfected Ba/F3 cells. The differences between the two kinases strongly suggests that this is an approach that can aid in understanding specific pathophysiological processes in leukemias associated with specific translocations. The approach provides starting points to further understand the role of BCR/ABL, TEL/PDGFRβ, and other leukemogenic oncogenes in transformation using a common, clonal cell background, Ba/F3. One clear point is that BCR/ABL potentiates proteins involved in motility whereas no evidence exists in our data that TEL/PDGFRβ has a similar effect. Thus proteomics can define differences in the molecular mechanism of action of oncogenes.

Acknowledgments—We thank Dr. D. Williams, University of Cincinnati College of Medicine, Cincinnati, OH for provision of the Rho N19 construct. The anti-stathmin antibodies were a gift from Dr. A. Sobel, INSERM, Paris, and the anti-gelsolin antisera was kindly given to us by Dr. D. Kwiatkowski, Harvard Medical School, Boston, MA.

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