BCR/ABL Induces Multiple Abnormalities of Cytoskeletal Function

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Abstract

The *BCR/ABL* oncogene causes human chronic myelogenous leukemia (CML), a myeloproliferative disease characterized by massive expansion of hematopoietic progenitor cells and cells of the granulocyte lineage. When transfected into murine hematopoietic cell lines, *BCR/ABL* causes cytokine-independence and enhances viability. There is also growing evidence that $p210^{BCR/ABL}$ affects cytoskeletal structure. $p210^{BCR/ABL}$ binds to actin, and several cytoskeletal proteins are tyrosine phosphorylated by this oncoprotein. Also, at least one aspect of cytoskeletal function is abnormal, in that the affinity of β1 integrins for fibronectin is altered in CML cells. However, isolated changes in β1 integrin function would be unlikely to explain the clinical phenotype of CML. We used time-lapse video microscopy to study cell motility and cell morphology on extracellular cell matrix protein–coated surfaces of a series of cell lines before and after transformation by *BCR/ABL*. *BCR/ABL* was associated with a striking increase in spontaneous motility, membrane ruffling, formation of long actin extensions (filopodia) and accelerated the rate of protrusion and retraction of pseudopodia on fibronectin-coated surfaces. Also, while untransformed cells were sessile for long periods, *BCR/ABL*-transformed cells exhibited persistent motility, except for brief periods during cell division. Using cell lines transformed by a temperature-sensitive mutant of BCR/ABL, these kinetic abnormalities of cytoskeletal function were shown to require BCR/ABL tyrosine kinase activity. Similar abnormalities of cytoskeletal function on fibronectin-coated surfaces were observed when hematopoietic progenitor cells purified by CD34 selection from patients with CML were compared with CD34 positive cells from normal individuals. Interestingly, α-interferon treatment was found to slowly revert the abnormal motility phenotype of *BCR/ABL*-transformed cells towards normal. The increase in spontaneous motility and other defects of cytoskeletal function described here will be useful biological markers of the functional effects of BCR/ABL in hematopoietic cells. (*J. Clin. Invest.* 1997. 100:46–57.) Key words: chronic myelogenic leukemia • cytoskeleton • cell motility • fibronectin • actin

Introduction

Chronic myelogenous leukemia (CML)[1] is a clonal myeloproliferative disorder that has been extensively studied as a model of neoplastic transformation of a stem cell. It is associated in > 95% of cases with a t(9;22) chromosomal translocation (Philadelphia chromosome) (1). Most patients with CML start with a chronic phase in which there is expansion of all myeloid cells, but this initial phase is eventually followed by blast crisis where there is an accumulation of myeloid or lymphoid blasts in association with new genetic mutations (2).

The biological effects of *BCR/ABL* have been remarkably difficult to define. CML progenitor cells are actively cycling, possibly more so than normal progenitor cells, but are entirely growth factor dependent for proliferation in vitro. In tissue culture, introduction of *BCR/ABL* into growth factor-dependent cell lines or murine marrow cells typically generates cell lines that evolve from growth factor hypersensitivity (3) to full factor independence, but again, the significance of this prominent tissue culture effect is unclear (4). Even modest hypersensitivity to growth factors is not a common feature of primary CML progenitor cells, except in more advanced stages of the disease (5). *BCR/ABL* may also affect the sensitivity of CML cells to apoptosis (6, 7), thereby altering the normal homeostasis and potentially contributing to an accumulation of myeloid cells.

There are a number of previous observations that suggest BCR/ABL could affect cytoskeletal function (8–10). First, BCR/ABL localizes in the cytoskeleton by binding to actin through a COOH terminus actin-binding domain, and several of the most prominent tyrosine kinase substrates for BCR/ABL are cytoskeletal proteins (8–11). This cytoplasmic location for BCR/ABL is believed to be important for transformation, since BCR/ABL mutants that fail to localize properly have reduced transforming activity (12). Further, there are several observations that suggest BCR/ABL disrupts the normal function of the cytoskeleton. CML cells have a diminished capacity to adhere to stromal layers (13) and to fibronectin or its proteolytic fragments (14), which has been ascribed to altered function of beta integrins. Further, CML cells have been reported to have increased adhesion to laminin and collagen type IV (14). These observations are important, as altered adhesion to extracellular matrix proteins could lead to premature release of CML cells from the marrow. However, recent studies suggest that the actual situation is more complex, since

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1. *Abbreviations used in this paper:* CML, chronic myelogenous leukemia; Ph, Philadelphia.

short-term (30 min) adhesion to fibronectin was found to be increased by BCR/ABL, while only longer term adhesion was decreased (15). Finally, studies from our laboratory and others have shown that BCR/ABL activates several signal transduction pathways that can be transiently activated in normal cells by adhesion, binding extracellular matrix proteins, or cross-linking of integrins with monoclonal antibodies. Taken together, these previous studies suggested the extent of BCR/ABL-induced abnormalities of cytoskeletal function may have been underestimated. In this study, we show that BCR/ABL induces multiple abnormalities of cytoskeletal function associated with motility.

Methods

Cells and cell culture. The murine immature hematopoietic cell line BaF3 was cultured at 37°C with 5% CO_2 in RPMI 1640 (Mediatech, Washington, DC) containing 10% (vol/vol) WEHI-3B–conditioned medium as a source of IL-3 and 10% (vol/vol) fetal calf serum (PAA Laboratories Inc., Newport Beach, CA). The *BCR/ABL* expressing cell lines BaF3.p210$^{BCR/ABL}$ and BaF3.p190$^{BCR/ABL}$ were generated by transfection with the pGD vector containing the p210$^{BCR/ABL}$ and p190$^{BCR/ABL}$ cDNAs (16) as previously described (17). BaF3 cells expressing BCR/ABL were cultured in RPMI 1640 medium with 10% fetal calf serum, but without any source of IL-3. The temperature-sensitive BCR/ABL expressing cell line BaF3 (ts-BaF3.p210) was obtained from Dr. L.M. Wiedemann (Institute of Cancer Research, London, UK) (18). At the nonpermissive temperature (39°C) ts-BaF3.p210 cells are phenotypically similar to BaF3 cells. At the permissive temperature (33°C), the BCR/ABL tyrosine kinase is activated, the cells become factor independent for viability and adhesion to fibronectin is transiently increased. ts-BaF3.p210 cells were maintained in RPMI 1640, 10% WEHI conditioned medium, and 10% fetal calf serum at 39°C with 5% CO_2. Murine NIH3T3 cells, NIH3T3 cells expressing p210$^{BCR/ABL}$, or both Grb2 and p210$^{BCR/ABL}$, (generated as described, Pear, W., unpublished observations) were maintained in DMEM, and 10% (vol/vol) fetal calf serum at 37°C with 10% CO_2.

Hematopoietic progenitor cells from CML patients or normal volunteers were enriched by fluorescence-activated cell sorting (FACS; Becton Dickinson Co., Mountain View, CA) using an antibody to CD34 conjugated to phycoerythrin (Caltag Laboratories, Burlingame, CA). The starting material was bone marrow aspirates obtained with informed consent using Dana-Farber Cancer Institute approved protocols. A Coulter Epics Elite ESP was used for sterile cell sorting (Coulter Instruments, Hialeah, FL).

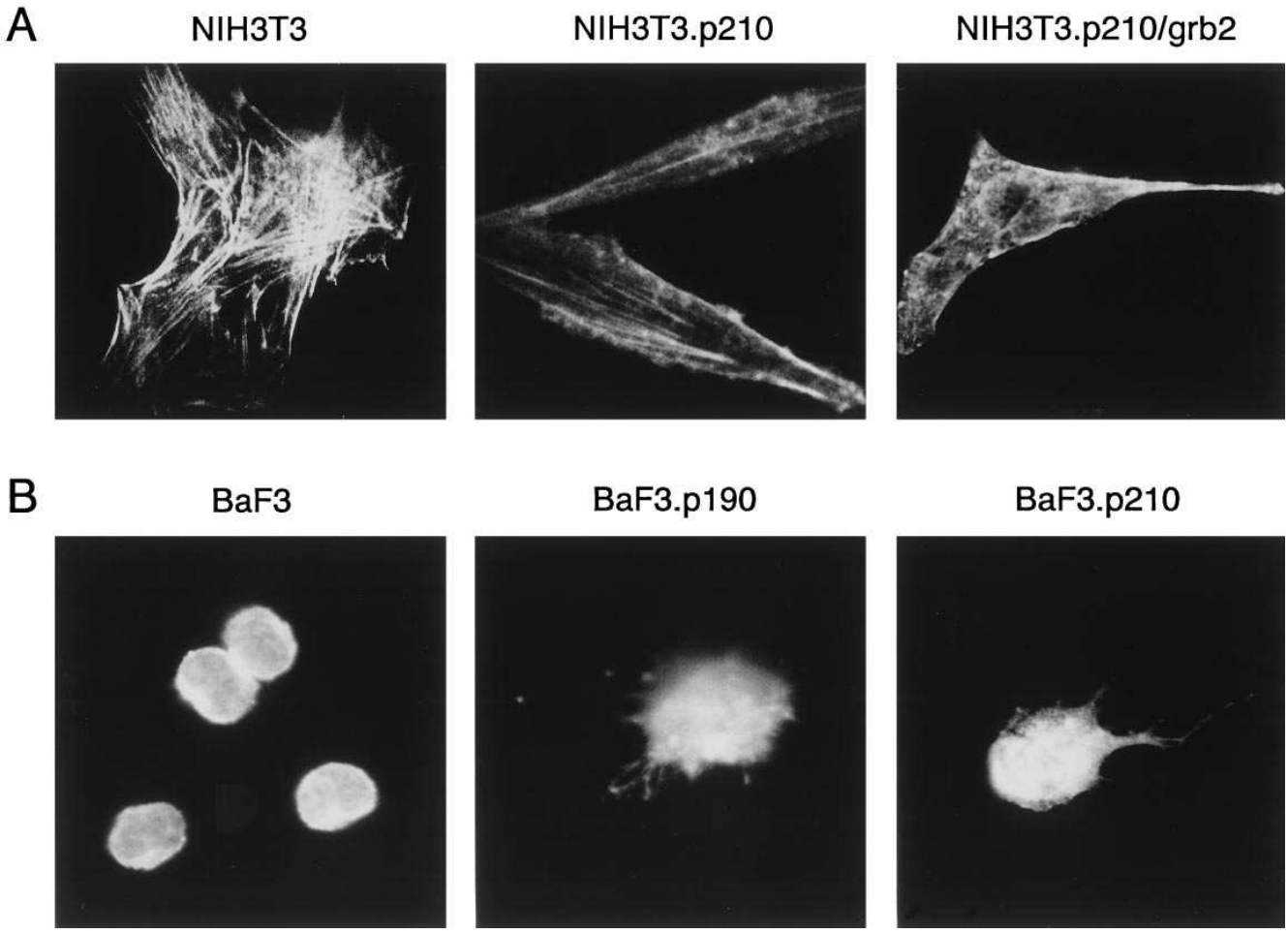

Figure 1. Differences in F-actin staining of untransformed and *BCR/ABL* transformed cells. Cells were fixed and stained for F-actin using rhodamine-labeled phalloidin. (*A*) Shown are NIH3T3 cells, NIH3T3 cells transfected by *BCR/ABL* (NIH3T3.p210), and NIH3T3 cells transformed by *BCR/ABL* and *Grb2* (NIH3T3.p210/grb2). (*B*) Shown are BaF3 cells, p190$^{BCR/ABL}$ transfected BaF3 cells (BaF3.p190), and p210$^{BCR/ABL}$ transfected BaF3 cells (BaF3.p210). The *BCR/ABL* cells have abnormal extensions containing F-actin.

Preparation of cell lysates and immunoblotting. Cell lysates were prepared and separated by 7.5% SDS-PAGE under reducing conditions, electrophoretically transferred to Immobilon PVDF (Millipore Corp., Bedford, MA) as described (19). The membranes were immunoblotted with mouse monoclonal antibodies against phosphotyrosine (1:1000, clone 4G10, courtesy of Dr. Brian Druker, Oregon Health Science University, Portland, OR), paxillin (1:5,000, clone 5H11 [20]), talin (1:2,000, clone 8d4; Sigma Chemical Co., St. Louis, MO), focal adhesion kinase (FAK) (1:500; Transduction Labs, Lexington, KY), and vinculin (1:2,000, clone VIN-11c, Sigma) using previously described methods (9, 19). Signals were detected using an enhanced chemiluminescense technique (Amersham, Arlington Heights, IL) (21).

F-actin staining of cells. F-actin was visualized in fixed cells (1% paraformaldehyde in phosphate-buffered saline) using rhodamine phalloidin (Molecular Probes, Eugene, OR) as previously described (9).

Adhesion assay of BaF3 cells. Adhesion of BaF3, BaF3.p190$^{BCR/ABL}$, and BaF3.p210$^{BCR/ABL}$ cells was measured as described (15) on plastic plates that were uncoated, coated with BSA, or fibronectin coated (Becton Dickinson Labware, Bedford, MA).

Time-lapse video microscopy. Cells were cultured on uncoated plastic tissue culture plates or plates coated with the extracellular matrix proteins fibronectin, collagen IV, collagen I, laminin, or vitronectinin (35-mm plates; Becton Dickinson Labware) in a temperature controlled chamber in their standard growth media. The cells were examined by video microscopy utilizing a Leitz inverted microscope (Diavert), Omega temperature control device, Hamamatsu C2400 video camera, MTI HR1000 TV, and Panasonic time-lapse S-VHS video recorder. The digital video images were captured and printed with a Kodak DS 8650 digital printer.

α-interferon treatment of cells. BaF3, BaF3.p210, ts-BaF3.p210 cells were plated onto fibronectin-coated plates and treated with mouse α-interferon (a gift from Dr. Glenn Dranoff, Dana-Farber Cancer Institute) using doses of 0–1,000 U/ml for 12 h and observed with time-lapse video microscopy for 24 h. CD34+ CML cells were also treated with 1,000 U/ml human α-interferon (Schering Corporation, Kenilworth, NJ) and observed with time-lapse video microscopy.

Results

BCR/ABL changes the actin cytoskeleton structure. F-actin was stained with rhodamine–phalloidin and examined using fluorescence microscopy (Fig. 1). The F-actin staining of NIH3T3 fibroblasts showed typical actin stress-fiber formation, whereas NIH3T3 cells transformed by *BCR/ABL* tended to be "spindle" shaped and had long cytoplasmic extensions containing actin ("filopodia"). Also, F-actin staining of *BCR/ABL*-transfected BaF3 cells (BaF3.p210, BaF3.p190) was brighter and the cells had altered morphology (containing abnormal extensions with actin) as compared with normal BaF3 cells. These results suggested that BCR/ABL affects the formation of actin-containing cell extensions and prompted us to examine the kinetics of formation of cell extensions such as filopodia and pseudopodia, and to look for abnormalities of motility.

Adhesion of BaF3 cells expressing BCR/ABL was measured on fibronectin, and compared to untransformed BaF3 cells. In a 6-h adhesion assay, as shown in Fig. 2, the BaF3.p210 and BaF3.p190 cells have similar adhesion as BaF3 cells on fibronectin; however, the *BCR/ABL*-expressing cells have nonspherical shapes.

BCR/ABL induces abnormalities of spontaneous motility on fibronectin-coated surfaces. Several cell lines expressing BCR/ABL were examined by time-lapse video microscopy, and compared to nontransformed cells. First, the effects of BCR/

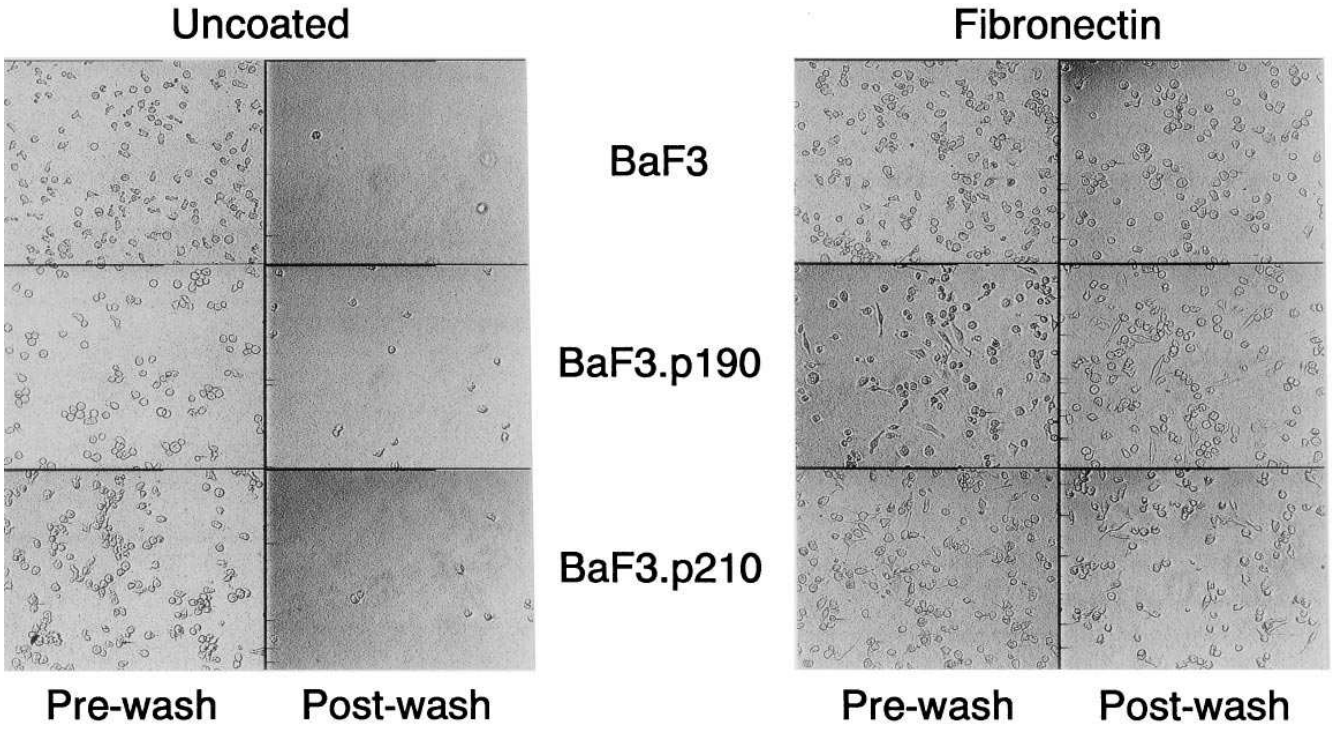

Figure 2. Adhesion assay of BaF3 and BCR/ABL expressing BaF3 cells on fibronectin. BaF3 cells, BaF3.p190$^{BCR/ABL}$, BaF3.p210$^{BCR/ABL}$ cells were plated onto uncoated (or BSA coated) and fibronectin-coated surfaces for 6 h and were photographed pre- and post-wash with PBS. On fibronectin surface, the *BCR/ABL* expressing cells have a nonspherical morphology.

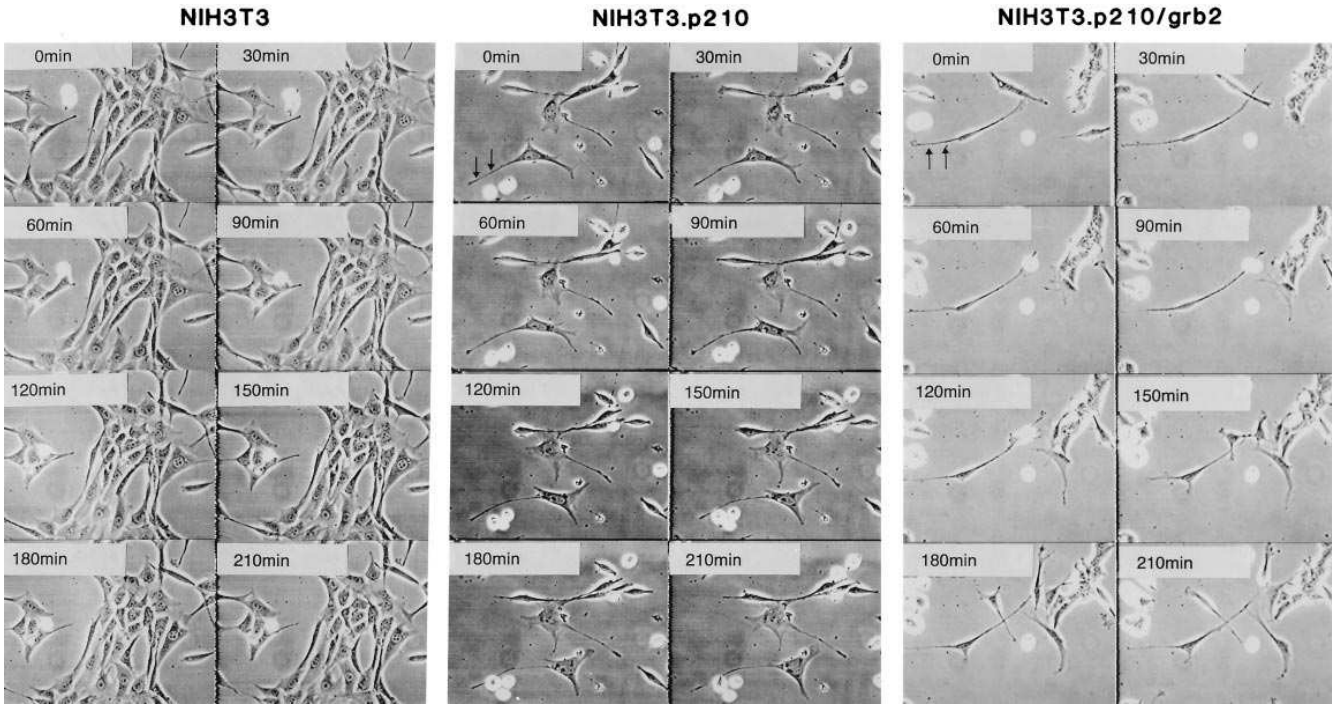

Figure 3. *BCR/ABL* expressing fibroblasts have abnormal cell motility. Shown are time-lapse video microscopy pictures, within the same field, at 30-min intervals. Cells utilized are NIH3T3 cells (*left panel*), NIH3T3.p210 cells (*middle panel*), and NIH3T3.p210/grb2 cells (*right panel*). Arrows point to the abnormal extensions containing actin.

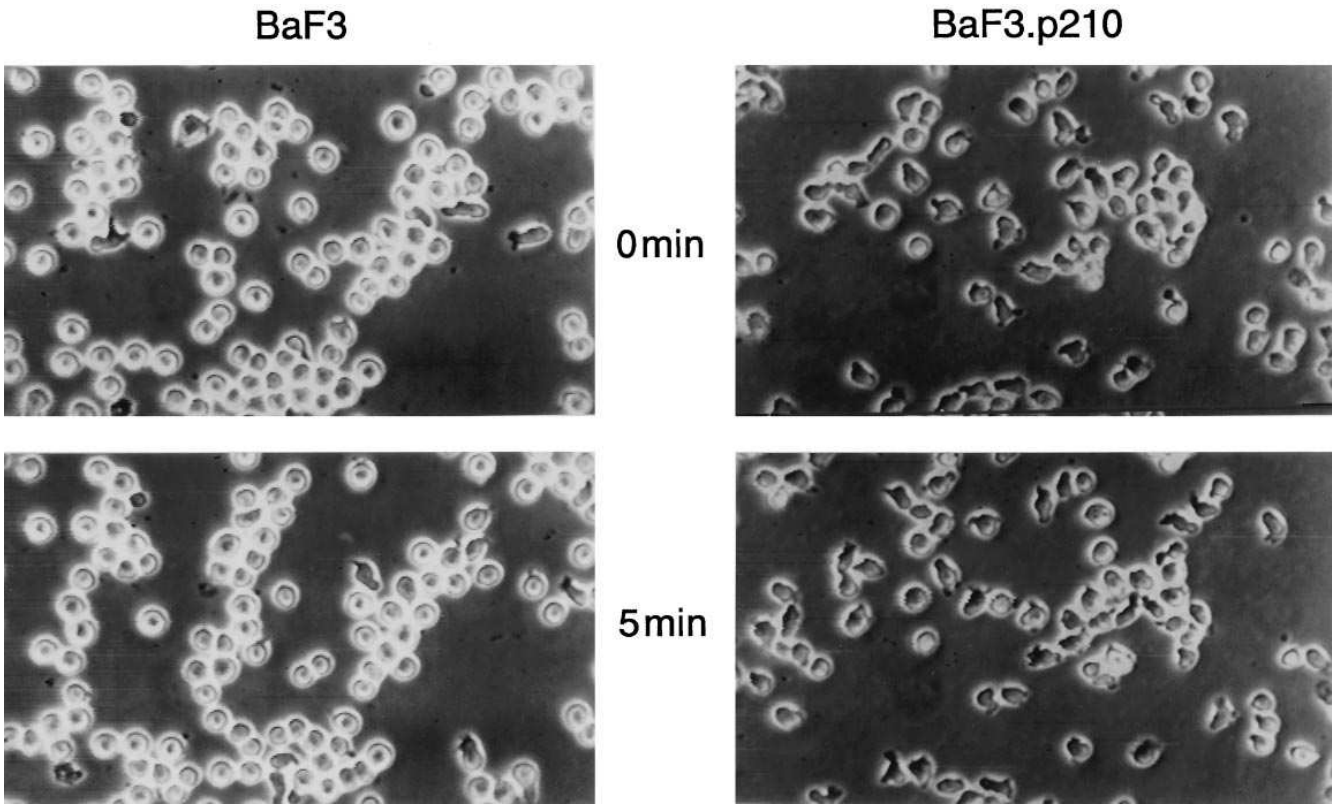

Figure 4. *BCR/ABL* expressing BaF3 cells have abnormal cell motility. Shown are time-lapse video microscopy pictures, within the same field, at a 5-min interval. Shown are BaF3 (BaF3) cells and BCR/ABL expressing BaF3 cells (BaF3.p210) on fibronectin-coated plates. The BaF3 cells are smaller than NIH3T3 cells, but much more motile (at least 6–10 times). Note the round morphology of normal BaF3 cells, and nonspherical morphology for BaF3.p210 cells (representing active protrusions/retractions, and ruffling). The *BCR/ABL* expressing cells are also strikingly motile compared with parental cells.

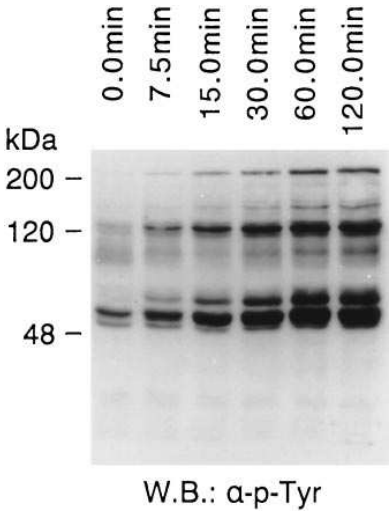

Figure 5. BaF3 cells expressing the temperature sensitive construct of *BCR/ABL* demonstrates the inducible tyrosine phosphorylation of several cellular proteins by the *BCR/ABL* oncogene. Growth factor-starved BaF3 cells stably transfected with a temperature sensitive *BCR/ABL* construct were transferred for the indicated times from the nonpermissive temperature (39°C) to the permissive temperature (33°C). Total cell lysates (2.5×10^5 cells) were applied to a 7.5% SDS-PAGE gel, transferred to PVDF-membrane, and immunoblotted with antiphosphotyrosine (4G10) antibody. Molecular mass is shown in kD.

ABL were examined in NIH3T3 cells transformed by BCR/ABL (NIH3T3.p210) or NIH3T3 cells made to express both $p210^{BCR/ABL}$ and Grb2 (NIH3T3.p210/grb2) (Fig. 3). Compared to untransformed NIH3T3 cells, the NIH3T3.p210 cells had abnormal filopodia extensions in ~ 10% of the population. Interestingly, the NIH3T3.p210/grb2 cells (which have a more transformed phenotype than NIH3T3.p210 cells) have many more filopodia, more lamellipodia and membrane ruffles, and an increased rate of spontaneous motility. The time-lapse photographs revealed that *BCR/ABL* expressing cells had a strikingly faster spontaneous migration rate than the untransformed fibroblasts. These differences were observed on fibronectin-coated as well as uncoated surfaces.

Using time-lapse video microscopy, untransformed BaF3 cells on fibronectin coated surfaces were found to be primarily rounded in shape, with little evidence of membrane ruffling or spontaneous motility. At any point in time, < 10% of cells had formed pseudopodia or other types of cell extensions, or were actively moving. In contrast, > 50% of BaF3.p210 cells cultured on fibronectin formed multiple pseudopodia or protrusions, with some of the cells having long extensions similar to filopodia (Fig. 4). Similarly, > 50% of cells were spontaneously moving and rarely had quiescent periods where they assumed a rounded morphology (with pseudopodia or other protrusions) and stopped moving. The exception was during mitosis, when all cells (BCR/ABL positive or negative) stopped migrating, became rounded and proceeded through cell division. BaF3.p210 cells also had an increased level of spontaneous membrane motion interpreted as membrane ruffling compared to untransformed cells.

The temperature-sensitive ts-BaF3.p210 cell line (18) is a

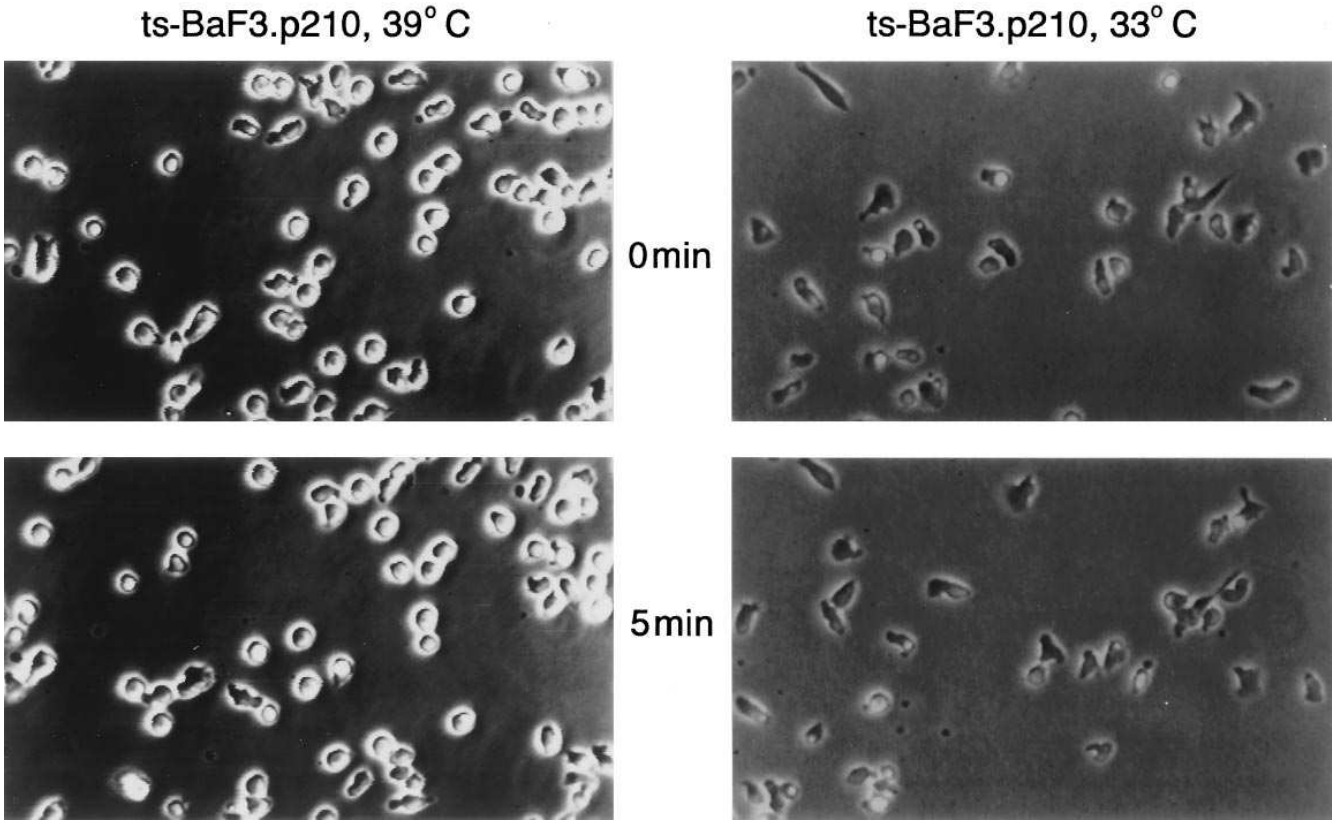

Figure 6. BCR/ABL causes abnormal cell motility in an inducible system. The ts-BaF3.p210 cells (which behave similar to parental BaF3 cells at nonpermissive temperature of 39°C, and as *BCR/ABL* expressing cells at permissive temperature of 33°C) on fibronectin-coated plates were utilized for time-lapse video microscopy. At nonpermissive temperatures, the cells have much less protrusion/retraction activity, and more spherical morphology as compared with cells at permissive temperatures. The cells at permissive temperatures also have abnormal actin extensions (including pseudopodia and filopodia).

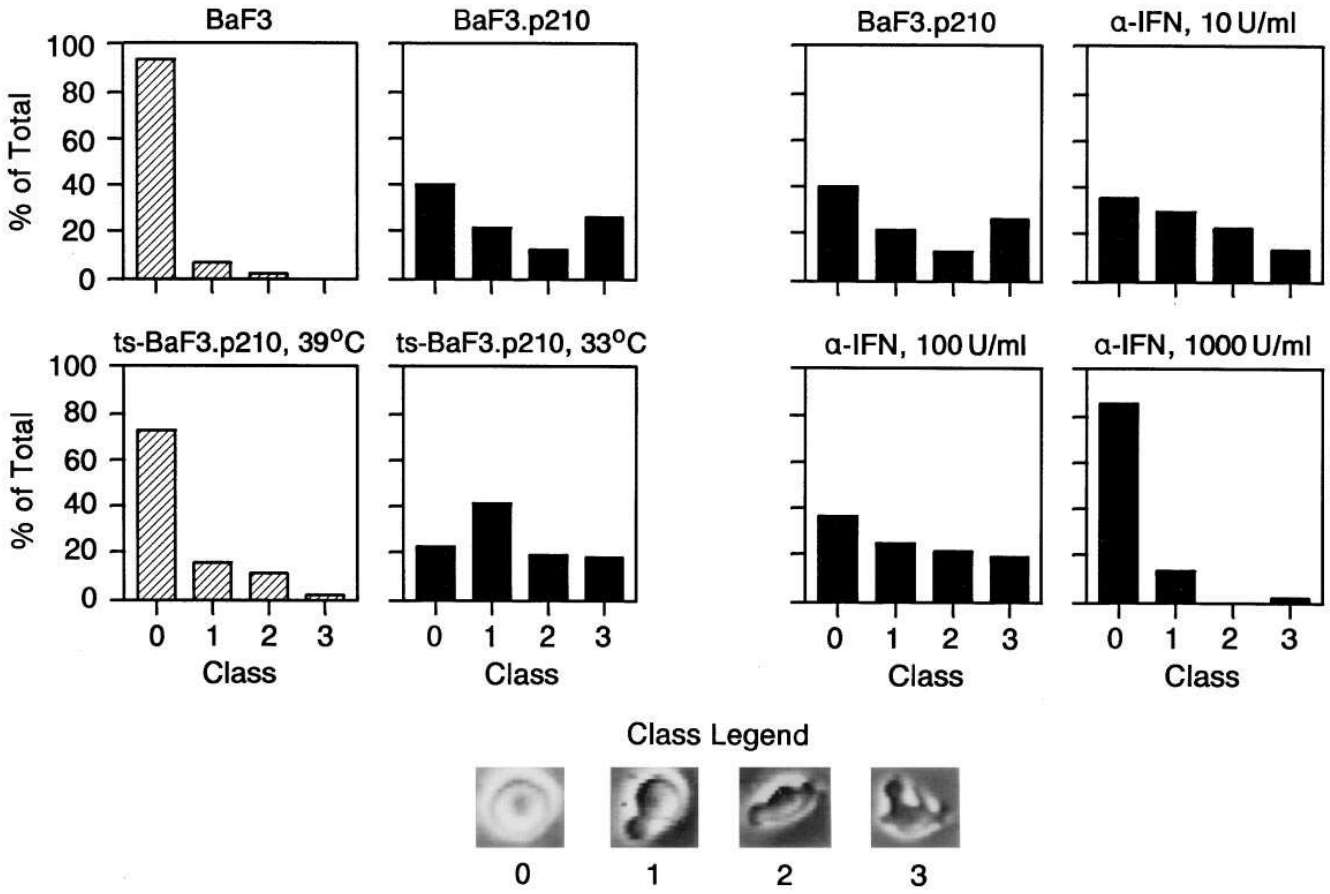

Figure 7. (*Left panel*) Quantitation of abnormal cell motility and cell morphology of *BCR/ABL* expressing BaF3 cells. Utilizing time-lapse video microscopy and transferring at least 10 different random frames onto the computer, cells were counted and categorized as follows: *0*, round morphology; *1*, having one protrusive structure; *2*, having two protrusive structures; *3*, having three or more protrusive structures. 300–500 cells were counted and categorized (by at least two different observers). These were then plotted onto a bar graph with the ordinate as the relative percentage of cells, and abscissa as the class of the cell. Appreciated are more BaF3.p210 cells in classes 1-3. (*Right panel*) Quantitation of abnormal cell motility and cell morphology of *BCR/ABL* expressing BaF3 cells in response to α-interferon. BaF3.p210 cells on fibronectin-coated plates were treated with an increasing amount of α-interferon (0–1000 U/ml) for at least 12 h, and time-lapse video microscopy performed for 24 h. The data from at least 10 different random frames were transferred onto the computer, cells were counted and categorized as above. Appreciated is the return to an apparent normal phenotype of BaF3.p210 cells treated with 1,000 U/ml α-interferon for 12 h.

useful model since at the nonpermissive temperature (39°C) these cells have low BCR/ABL tyrosine kinase activity and behave as untransformed BaF3 cells, and at permissive temperature (33°C) the BCR/ABL kinase is activated and phosphorylates several new cellular proteins (Fig. 5). On fibronectin plates, the ts-BaF3.p210 cells were similar to normal BaF3 cells at 39°C (the cells were mostly sessile, had a spherical morphology with few or no pseudopodia and exhibited slow movement), whereas, at 33°C they develop the increased motility and abnormal morphology of BaF3.p210 cells (with increased migration even on fibronectin surfaces, more disconcerted movement, and formation of multiple pseudopodia at the same time) (Fig. 6).

A relative scale was developed to quantitate the degree of cell shape change associated with BCR/ABL-transformation by assigning a grade from 0 (spherical morphology) to 3 (multiple pseudopodia and/or filopodia) (Fig. 7). Single frames were captured from the time-lapse videomicroscopy of cells on fibronectin-coated plates and 300–500 cells were evaluated. This scale provides a simple way to quantitate the degree of BCR/ABL-induced cell morphology changes and also provides a means of assessing the different degrees of motility observed from the video.

These studies indicate that BaF3 cells transformed by BCR/ABL have increased spontaneous motility on fibronectin-coated surfaces. The morphology and motility of BaF3 and BaF3.p210 cells on fibronectin-coated surfaces was then compared to that on uncoated surfaces and surfaces coated with other extracellular matrix proteins (collagen IV, collagen I, laminin, and vitronectin). It is interesting that the degree of spontaneous motility of the BaF3 cells was quite variable on the different surfaces. On collagen I, laminin, vitronectin, and uncoated plastic, BaF3 cells had considerably higher motility than they did on fibronectin or collagen IV, although the degree of motility did not reach that seen by BCR/ABL transformed BaF3 cells on the same surfaces. In contrast to BaF3 cells, BCR/ABL transformed cells were hyperactive on all of the surfaces (Fig. 8).

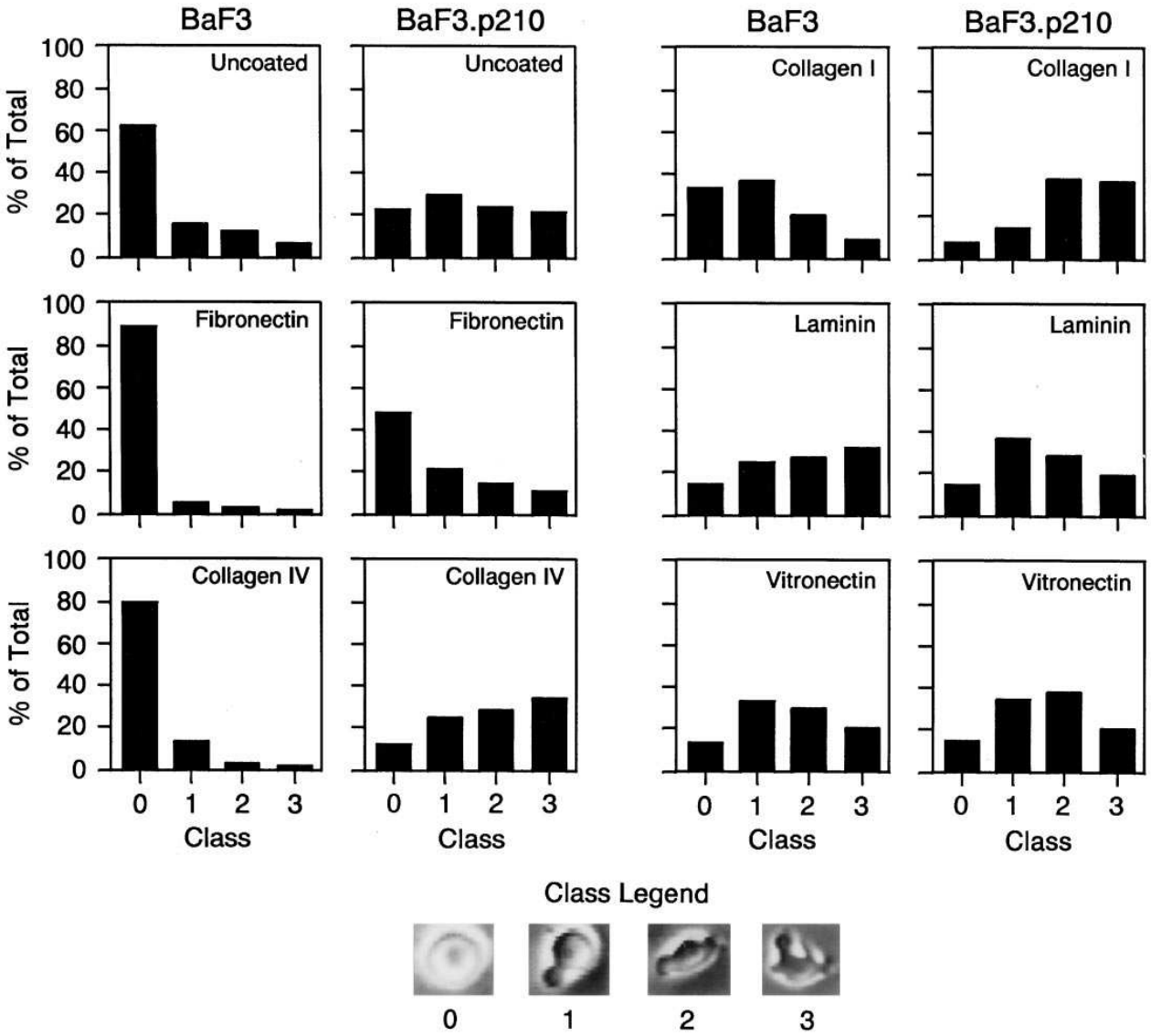

Figure 8. Quantitation of cell motility and cell morphology of *BCR/ABL* expressing BaF3 cells on various extracellular matrix protein surfaces. Utilizing time-lapse video microscopy and transferring at least 10 different random frames onto the computer from cells placed for 6 h on each surface, cells were counted and categorized as follows: *0*, round morphology; *1*, having one protrusive structure; *2*, having two protrusive structures; *3*, having three or more protrusive structures. At least 300–500 cells were counted and categorized (by at least two different observers). These were then plotted onto a bar graph with the ordinate as the relative percentage of cells, and abscissa as the class of the cell. The bar graphs show the effects of the various ECM surfaces. Appreciated are the effects of fibronectin and collagen IV on BaF3 cell morphology and motility.

α-interferon reduces cell motility and pseudopod formation of BCR/ABL expressing cells on a fibronectin-coated surface. α-interferon is useful in the therapy of patients with stable phase CML, although the mechanism of action is unknown. We examined the effects of α-interferon on the cell morphology and motility of normal and CML cells. α-interferon treatment (up to 1,000 U/ml) did not change the spontaneous motility of normal BaF3 cells (data not shown). However, treatment of BaF3.p210 cells with α-interferon (1,000 U/ml for 12 h) resulted in reduced spontaneous motility, reduced number of protrusions and pseudopodia per cell, and increased number of cells with spherical morphology (Figs. 7 and 9). Careful microscopic examination of *BCR/ABL*-transformed cell lines cultured with α-interferon shows that although they are no longer motile, they still have dramatic ruffling of the cell membrane. Thus, α-interferon normalizes one feature of BCR/ABL-induced cytoskeletal abnormality, but does not affect the increased overall degree of membrane ruffling activity.

The effects of α-interferon were also examined in the ts-BaF3.p210 cell line model on fibronectin surfaces. When α-interferon was added at the permissive temperature, the cells became less motile and reverted to the morphology and motility rate similar to the cells originally at the nonpermissive temperature (data not shown). When added at the nonpermissive temperature, α-interferon had no visible effect, but pre-

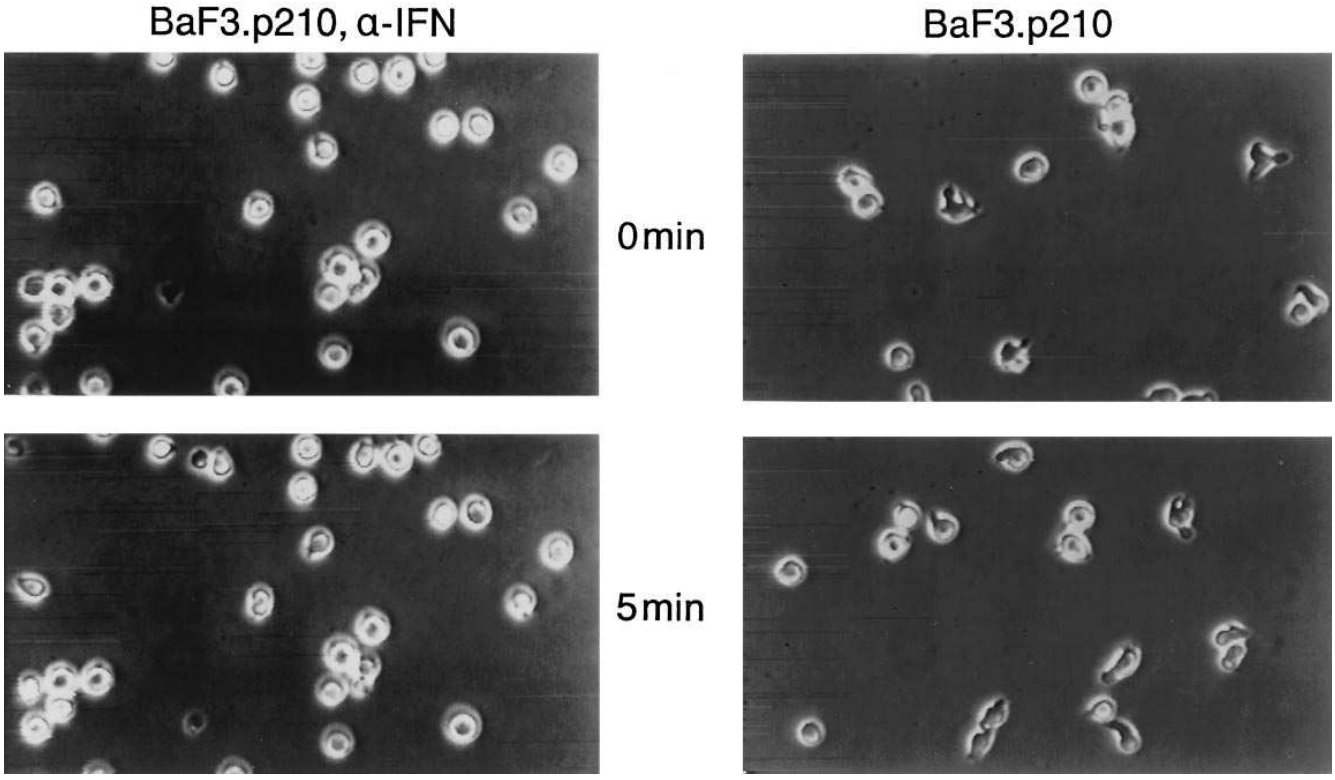

Figure 9. α-interferon retards hypermotility of *BCR/ABL* expressing cells and reverts the morphology of leukemic cells back to normal. Shown are time-lapse video microscopy pictures of cells on fibronectin-coated plates, within the same field, at a 5-min interval of BaF3.p210 cells (*right panel*) and BaF3.p210 cells treated with α-interferon at 1,000 U/ml (*left panel*). Note the multiple pseudopodia and distorted morphology of BCR/ABL-expressing cells, and the spherical morphology of α-interferon-treated cells.

vented the disordered motility of cells later switched to the permissive temperature.

Cell motility of CD34+ progenitor cells from CML patients is increased compared with normal controls on a fibronectin-coated surface. CD34+ progenitor cells were enriched by FACS from bone marrow samples from four patients with stable phase CML, and four normal subjects. The spontaneous motility and morphology of the CD34+ population from CML patients was different than normal progenitors as viewed by video microscopy on fibronectin-coated plates (Fig. 10). Shown in Fig. 10 are two representative samples from two normal and three CML patients. The CML cells, like the BaF3.p210$^{BCR/ABL}$ cells, have multiple pseudopodia and protrusions, and spontaneous motility of the cells was increased. A CML sample was also treated in vitro with α-interferon causing the cells to become more adherent to fibronectin and have a spherical morphology.

The focal adhesion protein paxillin is degraded in response to BCR/ABL. The results above show that BCR/ABL is associated with accelerated formation and retraction of pseudopodia and filopodia in BaF3 cells, NIH3T3 cells, and primary patient cells. We have previously shown that BCR/ABL phosphorylates a number of proteins associated with actin or focal adhesions, including tensin, talin, p125^{FAK}, vinculin, and paxillin (9). We asked if the increased rate of cytoskeletal rearrangement was also associated with degradation of any cytoskeletal proteins. In NIH3T3 cells transformed by BCR/ABL, there was accumulation of several lower molecular mass forms of paxillin (46 and 33 kD) which are recognized by an anti-paxillin monoclonal antibody we have previously generated (Fig. 11) (20). As previously shown, the anti-paxillin antibody (clone 5H11) is specific in recognizing paxillin and its degradation products (20).

We have demonstrated that the p46 and p33 forms are also tyrosine phosphorylated in *BCR/ABL*-transformed cells (data not shown) by immunoprecipitation studies. No significant accumulation of lower molecular weight forms of vinculin or talin was detected by immunoblotting in BCR/ABL transformed cells. Cells were also treated with α-interferon to determine if there was any accumulation of the p68 form of paxillin; however, there was no difference in the α-interferon treated cells with no treatment (data not shown).

Discussion

The Ph chromosomal translocation that causes CML fuses the c-ABL protooncogene from chromosome 9 to part of the BCR gene on chromosome 22. 5′ *BCR* exons typically join with the second exon of c-*ABL* to form *BCR/ABL* (22). The resulting fusion gene encodes a 210-kD oncoprotein in which the tyrosine kinase activity of c-ABL is increased by fivefold or more (23). This activation of c-ABL tyrosine kinase is essential for transformation of myeloid and lymphoid target cells (16, 24). An alternative breakpoint in *BCR* results in the formation

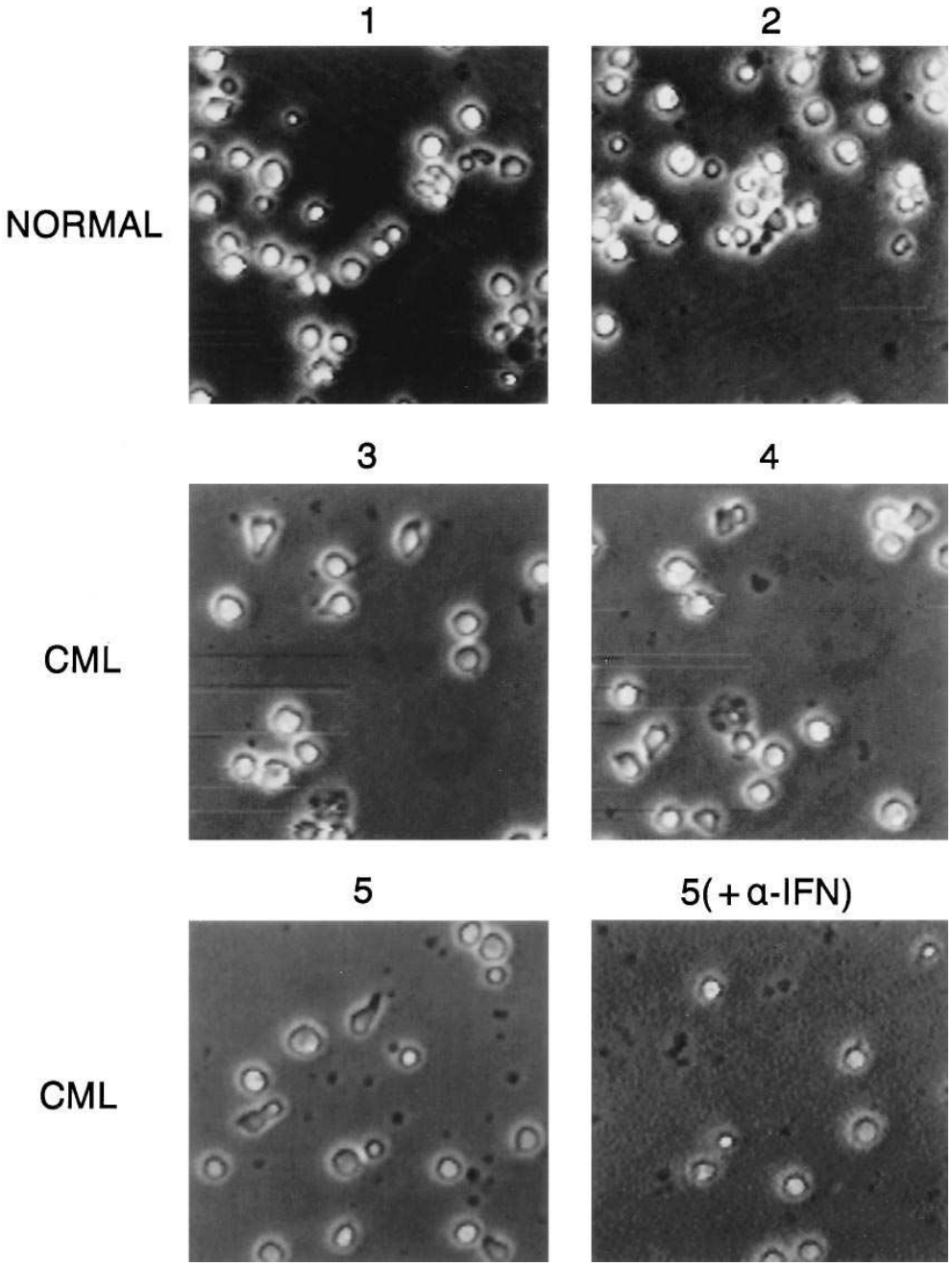

Figure 10. Cell motility of CD34+ progenitor cells from CML cells is abnormal, as compared to normal controls. Bone marrow samples from 3 patients with CML stable phase, and 2 normal volunteers, were obtained and CD34+ progenitor cells were isolated by FACS cell sorting after labeling with anti-CD34-PE antibody. On fibronectin-coated plates, the CD34+ population from CML stable phase patients was different than normal progenitors as viewed by time-lapse video microscopy. The CML cells, like the BaF3.p210 cells, were more migratory, and had multiple pseudopodia and filopodia. Also one CML CD34+ sample (5, 5+IFN-α) was treated with α-interferon and the abnormal motility with multiple pseudopodia and filopodia observed before treatment was reduced.

of a smaller fusion protein, $p190^{BCR/ABL}$, usually associated with Ph(+) ALL (25). The kinase activity of $p190^{BCR/ABL}$ is also elevated (26).

The mechanism whereby this oncogene transforms myeloid and lymphoid cells is unknown. Since the tyrosine kinase activity of ABL is required, it is likely that one or more kinase substrates exist that transmit signals from $p210^{BCR/ABL}$. It is interesting that the cellular localization of BCR/ABL is believed to be important for transformation, and at least 70% of the protein is localized to the cytoskeleton (27). Van Etten et al. (10, 11) and McWhirter et al. (8, 27) have identified an actin binding site at the COOH terminus of the ABL protein. In some assays of transformation by *BCR/ABL*, retention of this actin binding domain is required for full transforming activity. However, we have used immunofluorescence microscopy to show that $p210^{BCR/ABL}$ has a punctate peripheral staining pattern in addition to its actin localization, and further that proteins characteristic of focal adhesions, such as paxillin and vinculin, tend to colocalize with BCR/ABL in these punctate structures (9). Thus, our previous studies suggest that BCR/ABL accumulates in cytoskeletal structures that are likely to be involved in regulation of a variety of important cell functions, including binding to extracellular matrix proteins, cell motility, and cell morphology. The specific attachment sites of $p210^{BCR/ABL}$ within the cytoskeleton, in addition to actin, are currently unknown; although we have previously shown that BCR/ABL can coprecipitate with paxillin (19). In any event, modification of the actin cytoskeleton and cytoskeleton-associated proteins

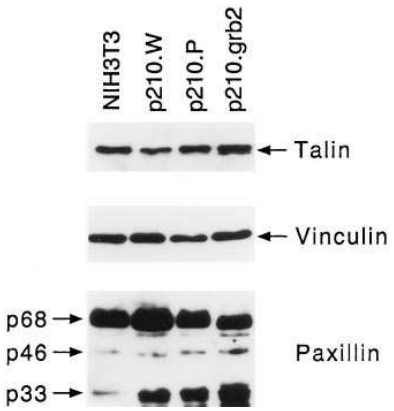

Figure 11. Expression of cytoskeletal proteins, and their degradation in *BCR/ABL* containing fibroblasts. Whole cell lysates (50 μg protein) of NIH3T3 cells, NIH3T3 cells transfected with *BCR/ABL* (two separate subclones: NIH.p210.W and NIH.p210.P) or NIH3T3 cells transfected with BCR/ABL-Grb2 (NIH.p210/grb2) were separated by 7.5% SDS-PAGE, transferred to PVDF-membrane, and probed with antibodies to talin, vinculin, or paxillin. Seen are at least three forms of paxillin in *BCR/ABL*-expressing cells (p46 and p33 probably being the degradation products of the original paxillin p68).

may be an important event in leukemogenesis (12). The results presented here suggest that the localization of BCR/ABL to the cytoskeleton results in more profound abnormalities of cytoskeletal function than were previously appreciated. Specifically, we observed increased staining for filamentous actin and an enhanced rate of formation and retraction of actin-containing protrusions such as pseudopodia and filopodia. Further, BCR/ABL-containing cells had increased level of spontaneous motility and much shorter periods of quiescence compared with nontransformed cells. These abnormalities required BCR/ABL tyrosine kinase activity and were observed in both *BCR/ABL*-transformed cell lines and primary CML progenitor cells isolated from patients with active leukemia.

The observed differences between untransformed and *BCR/ABL*-transformed cells were dependent on the surface to some degree. Untransformed BaF3 cells exhibited rounded morphology and little spontaneous motility on fibronectin and collagen IV, but higher motility and active pseudopod formation on laminin, collagen I, vitronectin, and uncoated plastic. The surface did not have any significant effect on BCR/ABL-transformed cells, which displayed active motility and pseudopod formation on all surfaces. These results can be interpreted in two ways. First, it is possible that BaF3 cells are inherently motile, and attachment to fibronectin and collagen IV send signals that inhibit motility. Second, BaF3 cells are inherently nonmotile, and laminin, collagen I, vitronectin, and uncoated plastic send signals inducing motility. In the first case, BCR/ABL would inhibit the down-modulating signals of fibronectin and collagen IV receptors. In the second case, BCR/ABL would send signals that lead to hypermotility. The results we have obtained so far do not allow a clear distinction between these possibilities, although the fact that *BCR/ABL*-transformed cells are more motile and morphologically aberrant compared to untransformed cells favors the latter hypothesis.

The significance of finding multiple abnormalities of cytoskeletal function may underlie some of the unusual clinical aspects of CML. In contrast to most other human leukemia oncogenes, *BCR/ABL* does not block differentiation, and does not appear to provide a major mitogenic signal. The major hallmarks of CML, early release of myeloid cells from the marrow and accumulation of myeloid cells at all stages of differentiation, are difficult to explain (2). One possibility is that the dysregulated motility observed here on certain surfaces may correlate with accelerated exit from the marrow of immature cells in CML patients (13), and it will be of interest to study motility on more complex surfaces such as marrow stromal cells. In the marrow microenvironment, which is likely to have abundant extracellular matrix proteins, the enhanced motility of CML cells could facilitate their early release and subsequent accumulation in other organs such as the spleen. The development of assays that can be used to study migration from the marrow would be helpful to further evaluate these hypotheses (28). Another possibility is that the hypermotility observed here is simply a manifestation of the abnormal activation of signal transduction pathways in the cytoskeleton by BCR/ABL (9, 19). The biological effects of such signaling activation may be profound. In most normal cells, signals from a variety of cell surface receptors are required to support growth and viability. In many cell types, the mitogenic signal from a growth factor receptor is insufficient, by itself, for normal cell proliferation and viability, and signals from adhesion or costimulatory receptors are also required (29). Such signals might indicate that the cell is located in the "proper" microenvironment, perhaps, or is in contact with other cells that are important for normal regulation of a particular lineage or tissue (28).

One interesting observation reported here is that a biologic therapy, α-interferon, long known to have significant therapeutic activity in CML (30) reduces some of the hypermotility of these cells. In the early 1980s α-interferon was shown to be beneficial for the therapy for CML. Randomized studies have suggested a survival advantage for patients treated with α-interferon compared with conventional chemotherapy (31). The time of progression to blastic phase is prolonged, and there is a higher rate of cytogenetic remission. Remarkably, although α-interferon is the only therapy other than bone marrow transplantation to induce cytogenetic remission, the mechanism of its action is not understood. At high concentrations, in vitro, α-interferon inhibits the proliferation of both normal and CML cells, but does not appear to be differentially more active on CML cells (30). It has recently been proposed that α-interferon may overcome the defective adherence of CML progenitors to stromal cells. α-interferon increases the expression of the adhesion molecule LFA-3, which is decreased in CML cells (32). Defects in β1 integrin function have been shown to be corrected by α-interferon (33). In our study, α-interferon treatment of *BCR/ABL*-expressing cells and CML patient samples reverted the abnormal motility and morphology to a more normal phenotype. It will be of interest to examine motility of cells from patients treated with α-interferon to look for in vivo effects of α-interferon that might correlate with our in vitro studies. Further, it may be of interest to compare the effects of α-interferon on cells from CML patients who are either sensitive or resistant to α-interferon therapy.

It will be important to determine the links between BCR/ABL and the cytoskeletal abnormalities described here. In preliminary studies, we have found that *BCR/ABL* induces accumulation of degradation products of the focal adhesion protein paxillin. The antibody for paxillin recognizes paxillin at 68 kD (p68), and also degradation products of p68 at 46 kD (p46) and at 33 kD (p33) (20). In vitro, similar lower molecular

weight forms of paxillin can be generated by proteolytic cleavage of paxillin by calpain II (34). These smaller molecular weight forms of paxillin may actually be signaling proteins since we have observed that the adapter protein CRKL ([35], and unpublished observations) and the protooncoprotein $p120^{CBL}$ (36) form a complex with p46 and p33 proteins to a much greater extent than with p68 paxillin itself. We have also observed the increased tyrosine phosphorylation of the p46 and p33 forms in CML hematopoietic cells (19). In any event, the apparent accumulation of low molecular weight forms of paxillin may be a biochemical marker for the disordered hypermotility of the BCR/ABL-transformed cell.

In summary, we show here that *BCR/ABL* globally disrupts the function of the cell machinery that interacts with the microenvironment. The outcome may be a cell with disordered motility that is also receiving false "signals" that indicate that certain adhesion receptors (such as integrins) are stimulated even in the absence of actual ligand binding. These cells still need hematopoietic growth factors for growth and viability, but may be able to migrate into capillaries at an immature stage of development, and proliferate in tissues where myeloid cells are not normally found. Overall, we suggest that the significant abnormalities of spontaneous motility we have found may be a fundamental abnormality of CML cells. If so, then future studies in which abnormalities of signal transduction pathways are analyzed in CML cells should be done with the view to determining their contribution to hypermotility as well as other biological functions ascribed to BCR/ABL.

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