[CANCER RESEARCH 56, 3426–3430, August 1, 1996]

Advances in Brief

P210 Bcr-Abl Interacts with the Interleukin 3 Receptor β_c Subunit and Constitutively Induces Its Tyrosine Phosphorylation[1]

Jeanne Wilson-Rawls,[2] Shanhai Xie, Jiaxin Liu, Pierre Laneuville,[2] and Ralph B. Arlinghaus[3]

Department of Molecular Pathology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030 [J. W-R., S. X., J. L., R. B. A.]; and Division of Hematology and Medical Oncology, Royal Victoria Hospital, McGill University, Montreal, Quebec H3A 1A1, Canada [P. L.]

Abstract

Chronic myelogenous leukemia is a neoplasm of pluripotent hematopoietic cells. The P210 Bcr-Abl oncoprotein is a deregulated cytoplasmic tyrosine kinase that has been shown to cause chronic myelogenous leukemia-like neoplasms in mice. Cytokines such as interleukin 3 and granulocyte/macrophage-colony-stimulating factor regulate the growth and differentiation of hematopoietic precursors. These cytokines activate two distinct signals to the nucleus. One signal is through the Ras pathway, and the second involves activation of Jak2. We demonstrated that Bcr-Abl co-immunoprecipitates with, and constitutively phosphorylates, the common β_c subunit of the interleukin 3 and granulocyte/macrophage-colony-stimulating factor receptors. Our data show that formation of this complex leads to the constitutive tyrosine phosphorylation of Jak2. It has been demonstrated that Bcr-Abl interacts with Grb2 and Shc, which in turn activates the Ras pathway. Our new findings raise the possibility that Bcr-Abl activates signaling through both pathways in a factor-independent fashion.

Introduction

CML[4] is a clonal disorder of pluripotent hematopoietic stem cells (1). CML has a biphasic clinical course. There is an initial chronic phase, which is marked by an increase in the number of mature myeloid cells, and increased proliferation of myeloid lineage cells. The chronic phase of the disease is unstable and progresses to an aggressive acute phase. The acute phase is characterized by the presence of blasts in the periphery that are unable to differentiate. The cytogenetic hallmark of CML is the Ph, which arises through a reciprocal t(9;22) translocation that results in the fusion of the *BCR* and *ABL* genes, generating a chimeric *BCR-ABL* oncogene, and the resultant oncoprotein (2). There are two forms of the Bcr-Abl oncoprotein, P210 and P185, depending on the precise site of the translocation within the *BCR* gene. Bcr-Abl is cytoplasmic and has deregulated Abl tyrosine kinase activity (3, 4). Transgenic or bone marrow transplanted mice expressing either form of Bcr-Abl generate hematopoietic neoplasms and CML-like diseases (5, 6). Bcr-Abl expression in IL-3-dependent cells will abrogate the factor requirement and prevent apoptosis, which is normally induced upon factor withdrawal (7). Recent studies have demonstrated that in fibroblasts and some Ph-positive cell lines, Bcr-Abl can activate the Ras pathway by interacting directly with the Ras activators Grb2 (8, 9) and Shc (9, 10). Thus, Bcr-Abl can activate signaling in a ligand-independent manner.

Hematopoiesis is tightly regulated by stromal interactions in the bone marrow and by cytokines. Most cytokines have a wide range of functions, and many have redundant activities so that they form a network of hematopoietic regulators (11). IL-3 and GM-CSF have overlapping effects on cells of the myeloid lineage, including inducing the proliferation and differentiation of early multipotent progenitors and inducing viability and enhancing the functional activity of mature myeloid cells. As myeloid cells differentiate from pluripotent bone marrow progenitors to mature cells, cytokines will elicit different responses through the same receptor signaling apparatus. The receptors for IL-3 and GM-CSF, and that of IL-5, form a family as they share a receptor subunit (β_c). In response to ligand binding to the IL-3 or GM-CSF receptors, several cellular substrates become phosphorylated on tyrosine residues, such as the β_c subunit (12), Jak2 (13), and Shc (14). Knowing that Bcr-Abl can activate the Ras pathway and transgenic animals develop only hematopoietic neoplasms (5, 6), it is logical that the cytokine signal pathways would be targets of the Bcr-Abl oncoprotein. Therefore, we used the human hematopoietic cell line MO7e (7), which is dependent on IL-3 or GM-CSF for proliferation, as a model system to examine the ability of Bcr-Abl to activate cytokine signal pathways.

The expression of Bcr-Abl in MO7e cells leads to factor-independent growth (7). Furthermore, these cells (termed M3.16) will no longer undergo apoptosis in the absence of exogenous factor (7). We demonstrate that Bcr-Abl interacts with the common β_c subunit of the IL-3 family of receptors and phosphorylates it on tyrosine. In addition, our results indicate that Jak2 is constitutively tyrosine phosphorylated in M3.16 cells. These results are in accord with recent data from Danial *et al.* (15), which demonstrated that v-*abl* caused constitutive Jak-Stat signaling in pre-B cells. Our findings provide important clues for determining how Bcr-Abl transforms hematopoietic cells. Perhaps understanding how Bcr-Abl interacts with the cytokine signaling machinery will also shed light on the mechanism of progression from the chronic phase to the acute phase in CML.

Materials and Methods

Cells. MO7e cells are human cytokine-dependent cells with a megakaryoblastic phenotype. The retroviral vector used for expression of P210 Bcr-Abl in the M3.16 cells has been described elsewhere (7).

Western Blots and Immunoprecipitations. For Western blots done using total cell lysates, 5×10^6 cells were washed twice in ice-cold PBS. Cell pellets were resuspended in hot 2× Laemmli buffer and boiled for 5 min. For immunoprecipitations followed by Western blot analysis, M3.16 or MO7e cells were serum starved, or serum and IL-3 starved, respectively, for 2–4 h. Cells were stimulated with either 10% FCS, 50 units/ml recombinant human IL-3 (Boehringer Mannheim; Genetics Institute), or recombinant human GM-CSF (Boehringer Mannheim) for 15 min at 37°C. Two $\times 10^7$ cells were lysed on ice 30 min in NTT or NTB lysis buffer [137 mM NaCl, 50 mM Tris, pH 7.5, 0.1% Triton X-100 or 1% Brij 98/99, 1 mM EDTA, 10% glycerol, 0.5 mM

Received 5/7/96; accepted 6/12/96.

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[1] This work was supported by Grants CA65611 and CA16672 from the NIH. R. B. A. is the recipient of the Hubert L. Stringer Chair in Cancer Research. J. W-R., a postdoctoral fellow, was supported by Grant CA09299. Initial findings were presented in November of 1995 at the XVIII International Symposium on Leukemia and Related Diseases in Kyoto, Japan.

[2] Present address: The University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Blvd., Dallas, TX 75235-9148.

[3] To whom requests for reprints should be addressed, at Department of Molecular Pathology, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030.

[4] The abbreviations used are: CML, chronic myelogenous leukemia; Ph, Philadelphia chromosome; IL-3, interleukin 3; GM-CSF, granulocyte/macrophage-colony-stimulating factor.

phenylmethylsulfonyl fluoride, 3 μg/ml aprotinin, 1 mM NaF, and 1 mM Na_3VO_4). Lysates were immunoprecipitated using antisera immobilized on protein A/G plus agarose (Santa Cruz Biotechnology). Proteins were resolved on 8% SDS-PAGE. The gels were equilibrated in transfer buffer [48 mM Tris, 39 mM glycine (pH 9.2), and 0.004% SDS (w/v)] and electroblotted to Immobilon-P (Millipore) at 200 milliamps for 1 h. Western blots were blocked in TBS containing 5% nonfat dry milk and 0.1% Tween 20 (v/v), unless the RC20H antibody was used, then they were blocked according to the manufacturer's directions. The blots were developed by enhanced chemiluminescence (ECL; Amersham).

Antibodies. 8E9 is an anti-Abl monoclonal antibody; it is used at a 1:10,000 dilution in TBSTM [TBS, 1% nonfat dry milk, 0.05% Tween 20 (v/v); Ref. 16]. The Western blots probed for phosphotyrosine were done with RC20H (Transduction Laboratories), an anti-phosphotyrosine monoclonal antibody conjugated to horse radish peroxidase (HRP), at a 1:2500 dilution. These blots were blocked and incubated in 10 mM Tris, pH 7.5, 100 mM NaCl, 0.1% Tween-20, 1% BSA. The Jak2 antibody, which is directed against residues 758–776 of murine Jak2 (Santa Cruz Biotechnology, Inc.), was used at 1:1000 dilution for Western blots in TBSTM, and 10 μg/lysate was used in immunoprecipitations. The anti-Jak1 antibody is directed against residues 785–804 of murine Jak1 (Santa Cruz Biotechnology, Inc.); this antibody was used at the same dilution as the anti-Jak2 antibody. Both the Jak1 and Jak2-specific antisera recognize the human, murine, and rat Janus kinases. The anti-β_c antibody is a monoclonal anti-human antibody to an unknown epitope (Santa Cruz Biotechnology, Inc.). These antibody treatments were followed by incubation of the blot with an appropriate anti-immunoglobulin-horseradish peroxidase conjugate (Amersham) at a 1:1500 to 1:2500 dilution, and development by ECL.

Kinase Assays. The M3.16 or MO7e cells were starved for 16 h, and the immunoprecipitations were done as described above. Bcr-Abl was immunoprecipitated with p6D, an anti-Abl monoclonal directed against amino acids 51–64 of human c-Abl (9). For COS-1 expression of Bcr-Abl, a cDNA clone in the pSG5 expression vector (Stratagene) was transiently transfected into the cells as described (16). After washing the immunoprecipitates with lysis buffer, the kinase assays were done using [γ^{32}P]ATP (DuPont NEN) as described (16). The phosphorylated proteins were resolved on 8% (Fig. 2*A*) or 6.5% (Fig. 2*B*) SDS-PAGE. The data was visualized by a phosphorimager.

Results and Discussion

Recent research has shown that Bcr-Abl can activate the Ras pathway by binding directly with Ras activators in transfected fibroblasts and some Ph-positive cell lines (8–10). Since the growth and differentiation of hematopoietic cells is tightly regulated by cytokines, we determined whether the cytokine signaling pathways might also be activated by Bcr-Abl. We used MO7e cells, a human megakaryoblastic cell line, which is dependent on exogenous IL-3 or GM-CSF for growth (7), as a model system to explore hematopoietic specific signaling. Expression of Bcr-Abl from a retrovirus vector in MO7e cells leads to factor-independent growth (7). Some of the Bcr-Abl-expressing clones that were isolated from transfected MO7e cells secrete low levels of GM-CSF in an autocrine fashion (7). However, the particular clone (M3.16) selected for these studies does not secrete functional levels of either GM-CSF or IL-3. For example, a 25× concentrate of M3.16 conditioned medium was unable to support growth of MO7e cells, unless rather large amounts of concentrate was added to the culture (7). Reverse transcription-PCR studies established that M3.16 cells express no detectable IL-3 RNA and only trace levels of GM-CSF RNA.[5]

Although there is evidence that Bcr-Abl interacts with Ras activators, there is no data to demonstrate that Bcr-Abl interacts with any of the cytokine or growth factor receptors. Neither chain of the IL-3 receptor has a catalytic domain; however, both are required for signal transduction (11). The β_c subunit is tyrosine phosphorylated in response to ligand binding (12). Although the β_c subunit does not bind to ligand, it is required for signal transduction. Therefore, we examined the phosphorylation state of the β_c subunit in the Bcr-Abl-expressing M3.16 cells. Starved M3.16 and MO7e cells were stimulated with IL-3, and the cell lysates were subsequently immunoprecipitated with a monoclonal antibody specific to the human β_c subunit (Santa Cruz Biotechnology, Inc.), and the blotted proteins were probed for phosphotyrosine. The identity of the β_c band was confirmed by stripping the blot and reprobing with a polyclonal anti-β_c antibody (Santa Cruz Biotechnology, Inc.; data not shown). The p140 β_c band was tyrosine phosphorylated in MO7e cells only when they were stimulated with IL-3 (Fig. 1*A*, compare lanes *c* and *d*). However, in M3.16 cells the β_c subunit was tyrosine phosphorylated in a constitutive manner (Fig. 1*A*, *lanes a* and *b*).

Comparing the phosphotyrosine proteins that co-immunoprecipitated with the β_c subunit (Fig. 1*A*, *lanes a* and *b*), there were a number of novel bands in the M3.16 cells. Most notable to us was an intense band of about the size of the Stat5 protein (M_r ~95,000); the other intense band (M_r ~55,000) is likely to be the heavy chain derived from the anti-βc immunoprecipitate. Also of interest is the band above the M_r 200,000 marker (Fig. 1*A*, *lanes a* and *b*, marked with an open *arrowhead*); this protein migrated at the approximate position of P210 Bcr-Abl. When the β_c immunoprecipitates were probed with an anti-Abl monoclonal antibody, it was evident that Bcr-Abl was complexed with the receptor chains, regardless of the activation state of the cell (Fig. 1*B*, *lanes b* and *c*). Normal mouse serum did not immunoprecipitate P210 Bcr-Abl (Fig. 1*B*, *lane a*). It is of interest that the normal Abl protein (P145) was not detected in the β_c subunit immunoprecipitations, suggesting that the Abl domain of Bcr-Abl is not responsible for the association with β_c. Thus, we conclude that Bcr-Abl co-immunoprecipitates with the common β_c subunit of the IL-3, GM-CSF, and IL-5 receptors. We do not know whether the association is direct (mediated by Bcr sequences?) or whether it is mediated by another protein.

To determine whether the IL-3 receptor β_c subunit was a target of the Bcr-Abl kinase, we did immune complex kinase assays. M3.16 cells were serum starved for 16 h, and Bcr-Abl and β_c were immunoprecipitated (Fig. 2). The immunoprecipitates were washed, and an immune complex kinase assay was carried out. The Bcr-Abl immunoprecipitate demonstrated a prominent P210 Bcr-Abl band (Fig. 2*A*, *lane a*). A band that co-migrated with Bcr-Abl was present in the β_c immunoprecipitate (Fig. 2*A*, *lane b*); however, this represented only a small proportion of the Bcr-Abl expressed in these cells. The P140 β_c band was also phosphorylated (Fig. 2*A*, *lane b*), appearing as a doublet. These results indicate that the IL-3 receptor β_c subunit could be a target of the Bcr-Abl kinase.

In the kinase assay done using proteins that have been co-immunoprecipitated with β_c from M3.16 cells, the complex will involve another tyrosine kinase, Jak1/Jak2. Jak2 is constitutively associated with the β_c subunit (17), and we have shown that it is tyrosine phosphorylated, and likely activated, in the presence of Bcr-Abl (Fig. 3). Therefore, it was possible that the phosphorylation of β_c could be entirely due to activated Jak2. So, we carried out an *in vitro* kinase assay using β_c and Bcr-Abl isolated from different sources to examine the ability of Bcr-Abl to phosphorylate the β_c subunit. P210 Bcr-Abl was expressed in COS1 cells and immunoprecipitated from cell lysates. The β_c subunit was immunoprecipitated from IL-3 and serum-starved MO7e cells, so that any co-precipitated Jak2 would be inactive. The immunoprecipitated proteins were mixed together, and the kinase assay was done. A protein the size of the β_c subunit was phosphorylated in the presence of Bcr-Abl only (Fig. 2*B*, compare *lanes a* and *b*). This indicated that the β_c subunit was likely a target of the Bcr-Abl tyrosine kinase. However, this result does not preclude the possibility that Jak2 is activated by tyrosine phosphorylation by

[5] P. Laneuville, unpublished observations.

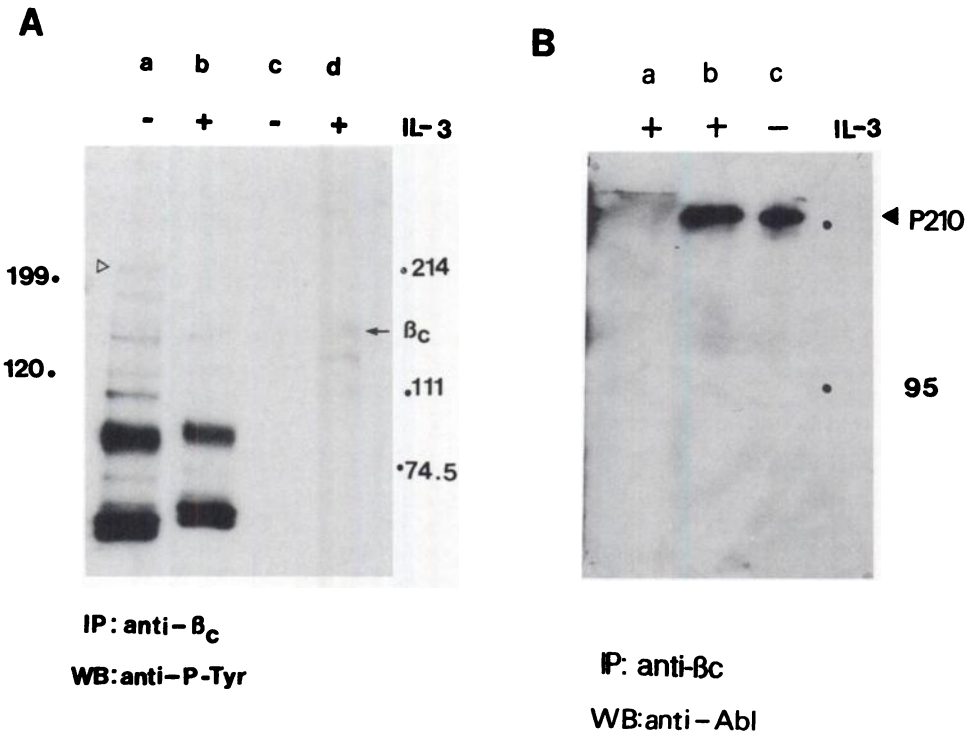

Fig. 1. The IL-3/GM-CSF/IL-5 receptor β_c subunit is phosphorylated constitutively and co-immunoprecipitates with Bcr-Abl. In *A*, the β_c subunit is constitutively phosphorylated in M3.16 cells. Cell lysates from M3.16 (*Lanes a* and *b*) and MO7e (*Lanes c* and *d*) cells were immunoprecipitated with a monoclonal antibody that recognizes the human β_c subunit (S-16; Santa Cruz Biotechnology, Inc.). The proteins were analyzed as described, and the anti-phosphotyrosine Western blot was done with RC20H. *Arrow*, the β_c subunit. *Open arrowhead*, a band that migrates at the same position as P210 Bcr-Abl. *B*, Bcr-Abl and the IL-3 receptor β_c subunit co-immunoprecipitate. To determine whether Bcr-Abl and the β_c subunit co-immunoprecipitate, M3.16 cells were treated as indicated in *A* and immunoprecipitated with either normal mouse serum (*Lane a*) or the S-16 anti-β_c antibody (*Lanes b* and *c*). The immunoprecipitates were examined by Western blotting with the anti-Abl 8E9 antibody. The band marked by a *closed arrowhead* comigrates with P210 BCR-ABL. Lanes and molecular weight markers are as indicated.

BCR-Abl in this assay and that Jak2 is responsible for the phosphorylation of the β_c subunit.

To determine whether Jak2 or Jak1 is tyrosine phosphorylated in M3.16 cells, we examined immunoprecipitates of these proteins by Western blotting with anti-phosphotyrosine antibody. We immunoprecipitated Jak2 from M3.16 cells and MO7e cells that had been serum or serum and IL-3 starved. Cells were then stimulated with 50 ng/ml of recombinant human IL-3 for 15 min at 37°C. Jak2 was immunoprecipitated from cell lysates with anti-Jak2 peptide antiserum (Santa Cruz Biotechnology, Inc.) and blotted. The Western blots were probed with an anti-phosphotyrosine antibody, RC20H (Transduction Laboratories), and the protein bands were visualized using ECL. Our results showed that Jak2 is constitutively tyrosine phosphorylated in M3.16 cells (Fig. 3*A*, compare M3.16 ± serum), whereas Jak1 was tyrosine phosphorylated in M3.16 cells only after serum stimulation (Fig. 3*B*, compare lanes *a* and *b*). To confirm the immunoprecipitation of Jak2, the blot was stripped and reprobed with the anti-Jak2 antibody (data not shown). In other experiments, we demonstrated that the immunoprecipitation of Jak2 was blocked by pretreatment of the antibody with excess peptide antigen (data not shown). We also showed that tyrosine phosphorylated Jak2 from M3.16 cells can be separated from tyrosine phosphorylated βc (data not shown). To rule out the possible autocrine effects of the extremely low cytokine production by M3.16 cells, we added 1 ng/ml of recombinant human GM-CSF to MO7e, which allows maximum growth (0.106 ng/ml permits one-half maximal growth[5]). Under these conditions, starved MO7e cells had only trace levels of tyrosine-phosphory-

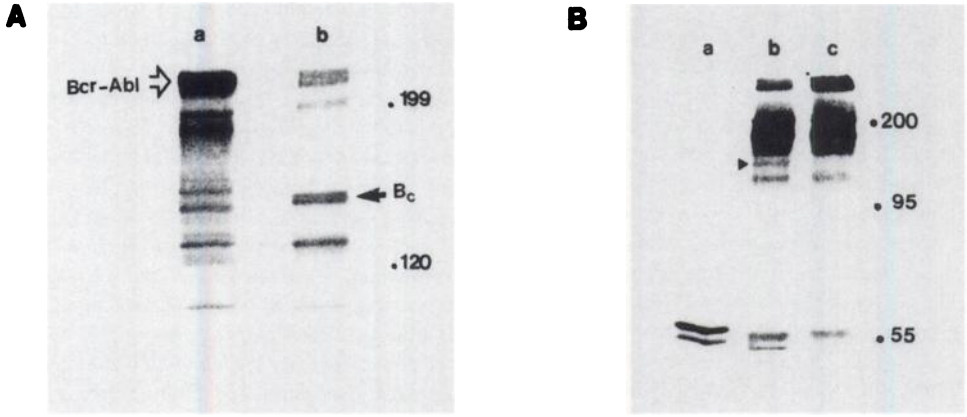

Fig. 2. The β_c subunit of the IL-3 receptor is a target of the Bcr-Abl kinase. *A*, Bcr-Abl can phosphorylate the β_c subunit. M3.16 cell lysates were immunoprecipitated with either anti-Abl p6D (*Lane a*) or the S-16 anti-β_c (*Lane b*) antibodies; the immunoprecipitates were washed, and the beads were resuspended in kinase buffer. The proteins were labeled with [γ^{32}P]ATP. The proteins were analyzed by SDS-PAGE, and the data were visualized by phosphorimager analysis. Bcr-Abl is identified by an *open arrow*. The β_c subunit is identified by a *solid arrow*. *B*, Bcr-Abl can transphosphorylate the β_c subunit. We immunoprecipitated the β_c subunit from MO7e cells that had been serum and factor starved for 16 h so that any associated Jak2 would be inactive. Bcr-Abl was immunoprecipitated from transfected COS1 cells (16). The immunoprecipitated proteins were washed and resuspended in kinase buffer. The kinase assays were carried out using the following combinations of proteins: β_c from starved MO7e cells only (*Lane a*), β_c and Bcr-Abl (*Lane b*), and Bcr-Abl only (*Lane c*). The proteins were analyzed on 6.5% SDS-PAGE. The band with the mobility of the β_c subunit is marked with a *solid arrowhead*. Lanes and molecular weight markers are as indicated.

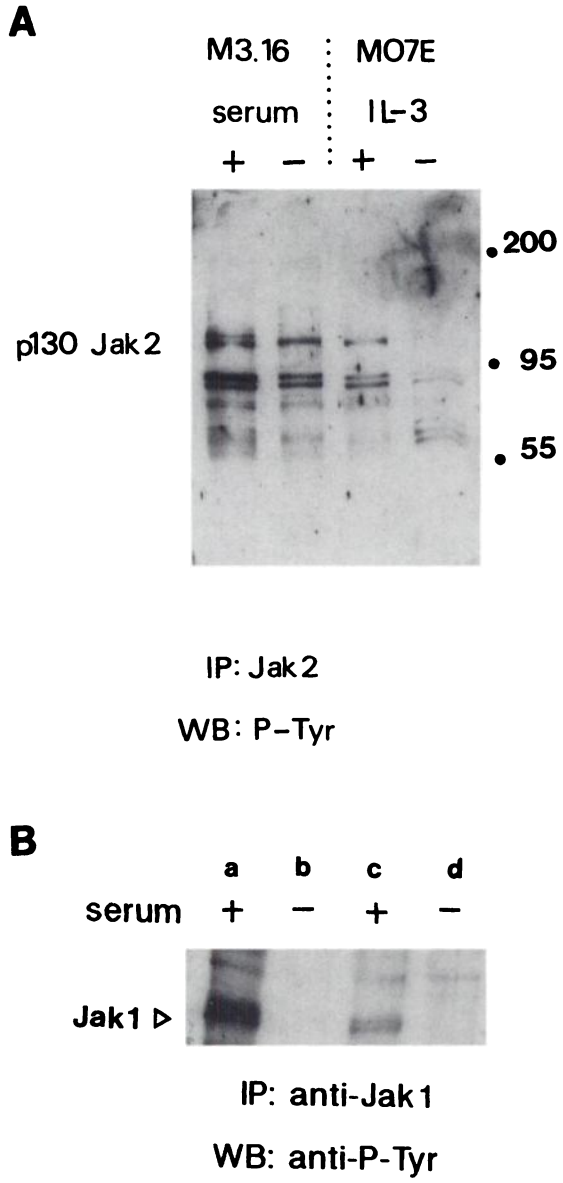

Fig. 3. Tyrosine phosphorylation of Jak2 is specifically induced by P210 BCR-ABL. To examine whether Bcr-Abl expression also activates Jak2 or Jak1, starved M3.16 cells were stimulated with 10% serum for 15 min at 37°C, and the phosphorylation state of Jak1 and Jak2 was compared. The cells were lysed in NTT buffer and analyzed as described previously. *A*, Jak2 is constitutively tyrosine phosphorylated in M3.16 cells. Jak2 was immunoprecipitated from M3.16 and MO7e cells as indicated. Immunoprecipitates were analyzed by anti-phosphotyrosine Western blotting. Lanes are as marked; prestained molecular weight markers (Bio-Rad) are as indicated. *B*, Jak1 is not activated by Bcr-Abl. We used an affinity-purified polyclonal antiserum directed against a peptide that corresponds to amino acids 785–804 of murine Jak1, HR-785 (Santa Cruz Biotechnology, Inc.) to demonstrate that Bcr-Abl does not activate Jak1 in myeloid cells, because Jak1 is only phosphorylated in response to serum in M3.16 cells (*Lanes a* and *b*) and MO7e (*Lanes c* and *d*) cells. Lanes are as indicated.

lated Jak2, suggesting that autocrine production of cytokines in M3.16 cells is not functionally significant (data not shown).

When IL-3 binds to its receptor, it activates two distinct signal pathways to the nucleus. Shc becomes phosphorylated on tyrosine and forms a complex with the β_c subunit of the receptor. Shc recruits the Grb2/SOS complex to the β_c subunit of the receptors in cytokine-stimulated cells and activates Ras (18). Jak2 is constitutively associated with the β_c subunit of the IL-3 receptor (17). In response to ligand binding, Jak2 is activated, and it signals to the nucleus through a Stat protein (18). It is known that Bcr-Abl can form complexes with Shc and Grb2 and activate the Ras pathway (8–10). In this report, we have shown that in addition to the abrogation of factor dependence (7), expression of P210 Bcr-Abl in MO7e cells can constitutively phosphorylate the β_c subunit and Jak2.

We demonstrated that Bcr-Abl formed a complex with the common β_c subunit of the IL-3 family of cytokine receptors (Fig. 1*B*). Furthermore, we showed that the β_c subunit was phosphorylated on tyrosine constitutively in the M3.16 cells (Fig. 1*A*) and was a target of the Bcr-Abl tyrosine kinase (Fig. 2). Since Jak2 is constitutively associated with the β_c subunit, it is likely that Jak2 is a part of a larger protein complex that includes Bcr-Abl. Our initial studies on Jak2 (Fig. 3) indicate that Jak2 but not Jak1 is constitutively tyrosine phosphorylated in Bcr-Abl-expressing M3.16 cells. Moreover, our experiments indicate that M3.16 cells do not produce functional levels of either GM-CSF or IL-3 (7).[5] These findings indicate that not only can Bcr-Abl activate the Ras pathway, but it can also interact with the β_c subunit inducing its tyrosine phosphorylation, and possibly activating Jak2 and signaling through Stat intermediates in a ligand-independent manner.

In normal hematopoietic maturation, IL-3 and GM-CSF stimulate the proliferation of early myeloid progenitors but also stimulate the differentiation of later committed myeloid lineage cells. If Bcr-Abl is activating the signaling pathways from these receptors in a ligand-independent manner, it could mimic some of the normal events in hematopoiesis but without the normal environmental controls. For example, expression of Bcr-Abl in early progenitors could activate the mitogenic response normally elicited by IL-3 and GM-CSF. However, this proliferation would be unregulated. Thus, it might be possible that a cell with the Ph translocation may divide many more times than a normal precursor in the bone marrow. Or during differentiation, the Ph-positive cells may divide at later stages of maturation than normal cells. In either case, this would result in an increase in the number of mature myeloid cells. This would be consistent with the clinical profile of the chronic phase, which is marked by increased numbers of mature myeloid cells.

M3.16 cells that express Bcr-Abl, although no longer factor dependent, have been shown to be further along the myeloid differentiation pathway than the parental cells (7). Thus, it is possible that Bcr-Abl expression in hematopoietic cells may induce the chronic phase of CML through interactions with IL-3 or GM-CSF signaling pathways. Consistent with this, it has been demonstrated that mice transplanted with bone marrow stem cells infected with a retrovirus that expresses IL-3 develop a myeloproliferative disorder that mimics the chronic phase of CML, *i.e.*, increased granulocytes in the periphery, but without the subsequent progression to a state where differentiation does not occur (19).

As a Ph-positive cell differentiates and changes its pattern of gene expression, stimulation of the same IL-3 receptor signaling apparatus by Bcr-Abl would elicit new responses. For example, expression of Bcr-Abl in several IL-3-dependent cell lines has also been demonstrated to abrogate apoptosis (20). Possibly, the activation of the IL-3-induced signaling pathways by Bcr-Abl in more mature cells activates not mitogenesis but protection from programmed cell death. Prolonged cell survival would also increase the number of mature myeloid cells in the periphery.

Acknowledgments

We thank Tammy Trlicek for assistance in manuscript preparation.

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