Coupling between p210bcr-abl and Shc and Grb2 Adaptor Proteins in Hematopoietic Cells Permits Growth Factor Receptor–independent Link to Ras Activation Pathway

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Summary

Enforced expression of p210bcr-abl transforms interleukin 3 (IL-3)–dependent hematopoietic cell lines to growth factor–independent proliferation. It has been demonstrated that nonreceptor tyrosine kinase oncogenes may couple to the p21ras pathway to exert their transforming effect. In particular, p210bcr-abl was recently found to effect p21ras activation in hematopoietic cells. In this context, experiments were performed to evaluate a protein signaling pathway by which p210bcr-abl might regulate p21ras. It was asked whether Shc p46/p52, a protein containing a src-homology region 2 (SH2) domain, and known to function upstream from p21ras, might form specific complexes with p210bcr-abl and thus, possibly alter p21ras activity by coupling to the guanine nucleotide exchange factor (Sos/CDC25) through the Grb2 protein–Sos complex. This latter complex has been previously demonstrated to occur ubiquitously. We found that p210bcr-abl formed a specific complex with Shc and with Grb2 in three different murine cell lines transfected with a p210bcr-abl expression vector. There appeared to be a higher order complex containing Shc, Grb2, and bcr-abl proteins. In contrast to p210bcr-abl transformed cells, in which there was constitutive tight association between Grb2 and Shc, binding between Grb2 and Shc was Steel factor (SLF)–dependent in a SLF-responsive, nontransformed parental cell line. The SLF-dependent association between Grb2 and Shc in nontransformed cells involved formation of a complex of Grb2 with c-*kit* receptor after SLF treatment. Thus, p210bcr-abl appears to function in a hematopoietic p21ras activation pathway to allow growth factor–independent coupling between Grb2, which exists in a complex with the guanine nucleotide exchange factor (Sos), and p21ras. Shc may not be required for Grb2–c-*kit* interaction, because it fails to bind strongly to c-*kit*.

Chronic myelogenous leukemia (CML)[1] is a malignancy of pluripotential hematopoietic stem cells that is characterized cytogenetically by the presence of the Philadelphia chromosome (Ph^1) (1, 2). This Ph^1 translocation results in the head-to-tail fusion of 5′ exons of the breakpoint cluster region (BCR) gene on chromosome 22 with the *abl* gene on chromosome 9 (3). This hybrid gene codes for a fusion protein (p210bcr-abl) with a dysregulated tyrosine kinase activity (4, 5). p210bcr-abl has been shown to transform lymphoid cells (6), to transform IL-3–dependent cell lines to growth factor–independent proliferation (7), to cause various hematopoietic neoplasms including human CML-like syndrome in mice (8, 9), and to lead to clonal heterogeneity in vitro (10). Thus, p210bcr-abl has been linked as a decisive inciting factor in the pathogenesis of CML. Therefore, determining the intracellular pathways p210bcr-abl uses to confer these phenotypes is of significant interest.

Discovery of a mutant H-ras with preferential affinity for GDP made possible a significant advance in understanding how ras proteins participate in mitogenic signaling (11). When expressed in mammalian cells stimulated to proliferate by activation of a tyrosine kinase receptor or by a nonreceptor tyrosine kinase oncogene, this mutant ras protein blocked transmission of tyrosine kinase proliferative signals (12, 13). We recently observed that the nonreceptor tyrosine kinase oncogene p210bcr-abl effects a high state of activation in the

[1] *Abbreviations used in this paper:* BCR, breakpoint cluster region; SH2, src-homology region 2; SLF, Steel factor; Sos, son of sevenless.

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normal cellular p21ras of hematopoietic cells (14). In these cells, p21ras activation, evidenced by GTP loading, was regulated by the tyrosine kinase activity of p210bcr-abl in the absence of guanine nucleotide pool changes (14).

It appears p210bcr-abl may activate p21ras by a mechanism facilitating guanine nucleotide exchange (e.g., by increasing the off-rate of GDP), based on estimates of guanine nucleotide pool size (intracellular ratios of GTP/GDP) and kinetics (15). This concept is consistent with the observation that the dominant negative inhibitory ras mutant (Asn17) reverses transformation of NIH3T3 cells caused by nonreceptor tyrosine kinase oncogenes (13). Dominant negative (Asn17) H-ras competitively inhibits the guanine nucleotide exchange factor, GDP dissociation stimulator (GDS), CDC25/Son of sevenless (Sos), in mitogenic signaling (16). Further, the lack of binding by p120 rasGAP to GDP-bound p21ras makes rasGAP an unlikely candidate for the upstream component targeted by dominant negative ras (15).

In this regard, the information provided by introducing dominant negative ras into cells complements those genetic studies performed in Drosophila and in the nematode *Caenorhabdtis elegans*, which place ras proteins downstream from tyrosine kinase receptors that are important for development (17–19). Coupling between tyrosine kinase receptors and p21ras has been linked, first, by these genetic studies, and more recently, by direct biochemical testing to Sem5/Grb2 (20–26). Grb2 contains a src-homology region 2 (SH2) domain that binds phosphotyrosine residue(s) of tyrosine kinase receptors (20), and two SH3 domains that are now known to bind to a proline-rich motif of the guanine nucleotide exchange factor Sos/CDC25 (21–23, 25, 26). The association between tyrosine kinase receptor and Grb2 was dependent upon ligand-activated tyrosine autophosphorylation of the receptor, whereas complex formation between Grb2 and Sos is independent of external signals (22).

Recently, a novel transforming protein called Shc, which contains a SH2 domain was cloned. By structural analysis, this protein lacks obvious catalytic domains to account for its transforming ability in NIH3T3 cells (27). Evidence exists functionally linking Shc to the ras signaling pathway (27, 28). Although Shc proteins are heavily phosphorylated on tyrosine in rat-2 cells transformed by v-src or v-fps, there is no evidence that Shc associates with these nonreceptor tyrosine kinases (29). On the other hand, Shc and Grb2 have been shown to bind directly to certain activated, autophosphorylated tyrosine kinase receptors with their SH2 domain (20, 27, 28). In addition, when Shc proteins are tyrosine phosphorylated, they bind with Grb2, which constitutively complexes with the guanine nucleotide exchange factor (Sos) (21, 23).

In this study, we examined how p210bcr-abl may lead to p21ras activation, and found evidence for a tight complex formed constitutively between p210bcr-abl, Shc, and Grb2 in p210bcr-abl transformed cells. In contrast, in a parental cell line lacking p210bcr-abl, but expressing the c-*kit* receptor, the association of Shc and Grb2 was dependent upon steel factor (SLF) stimulation. SLF triggered binding of Grb2 to tyrosine phosphorylated c-*kit*.

Materials and Methods

Cytokines and Antibodies. Highly purified recombinant murine steel factor (SLF) was a gift from Dr. Douglas E. Williams (Immunex Corp., Seattle, WA). Rat anti-c-*kit* mAb, ACK-2, which recognizes the extracellular domain of murine c-*kit*, was obtained from Gibco BRL (Gaithersburg, MD). Affinity-purified anti-Shc Ab (raised against a 10–12-kD protein fragment of Shc), anti-Shc mAb (SC73), affinity-purified anti-Grb2 Ab (raised against the entire 24-kD protein from rat brain), anti-Grb2 mAb (GR81), and r-antiphosphotyrosine Ab (RC20), were obtained from Transduction Laboratories (Lexington, KY). Mouse antiphosphotyrosine mAb (4G10) was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-ABL mAb (24-21) and polyclonal anti-BCR antibody were purchased from Oncogene Science, Inc. (Uniondale, NY).

Cells and Cell Culture. The 32DC13 cell line was obtained from Joel Greenberger (University of Massachusetts Medical Center, Worcester, MA). The NSF/N1.H7 cell line and the MMTVras.G5 cell lines have been described elsewhere (14, 30). The MMTVras.G5 cell line was derived by placing nonadherent bone marrow cells into McCoy's 5A modified medium (Gibco BRL) supplemented with 15% FCS and 10% WEHI-3 conditioned medium (a rich source of IL-3) plus SLF. These bone marrow cells were from the MMTV-v-H-ras transgenic oncomouse purchased from DuPont Co. (Wilmington, DE). Expression of the v-H-ras transgene was dependent upon the addition of dexamethasone. These cell lines were cultured in McCoy's 5A modified supplemented with 15% FCS and 10% WEHI-3 conditioned media. RmuSLF (50 ng/ml) was added for growth of the MMTVras.G5 cell line. K562 cell line supplied from the American Type Culture Collection (Rockville, MD) was cultured in RPMI 1640 supplemented with 10% FCS at 37°C, 5% CO_2. For experiments including factor stimulation, exponentially growing MMTVras.G5 cells and transformed derived MMTVras.bcr-ablG7 cells were washed and incubated for 18 h at 37°C in serum-free McCoy's 5A modified medium containing 0.5% BSA (Sigma Chemical Co., St. Louis, MO). Then cells were washed once with serum-free medium and exposed to growth factor.

DNA Transfection. The plasmid vector pGD210 that contains p210bcr-abl cDNA was obtained from Drs. G. Daley and D. Baltimore (The Rockefeller University, New York [8]). pGD210 was linearized with Ndel (New England Biolabs, Beverly, MA) before electroporation. Beginning 48 h after electroporation, cells were selected with G418. Within 10 d of plating in the selection medium, G418-resistant sublines were obtained.

Immunoblotting and Immunoprecipitation. Immunoblotting was performed as previously described (31). Cells were lysed in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X (Bio-Rad Laboratories, Richmond, CA) 1 mM PMSF, 0.15 U/ml aprotinin, 10 μg/ml leupeptin, 100 mM sodium fluoride, and 2 mM sodium orthovanadate (Sigma Chemical Co.). Cell lysates at 1 mg/100 μl were mixed 1:1 with SDS sample buffer containing 2-ME, boiled for 5 min, and separated by SDS-PAGE. Proteins were transferred onto Immobilon P membrane (Millipore Corp., Bedford, MA). Immunoblots were blocked in TBST (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) containing 1% BSA for 1 h. The blots were incubated with the appropriate primary Abs (antiphosphotyrosine mAb (4G10), 1:1,000; r-antiphosphotyrosine Ab (RC20), 1:2,500; anti-Shc mAb, 1:250; anti-Grb2mAb, 1:250; anti-ABL mAb, 1:200 dilution) in TBST containing 1% BSA) for 2 h at room temperature. Detection of the primary Ab was done with either one or two commercially available kits: (*a*) secondary Ab conjugated to horseradish peroxidase (Amersham,

Arlington Heights, IL) at 1:2,000 dilution and later developed by chemiluminescent reaction and exposed to radiographic film; or (*b*) secondary Ab conjugated to alkaline phosphatase (Zymed Laboratories, Inc., S. San Francisco, CA) at 1:1,000 dilution and developed with substrate, resulting in a visible color reaction on the membrane. Immunoprecipitations were performed by incubating cell lysates with appropriate Ab for 2 h at 4°C. Anti-Shc, anti-Grb2, and anti-BCR immunoprecipitates were collected by protein A-Sepharose beads (Pharmacia, Uppsala, Sweden). Anti-ABL and anti-c-*kit* immunoprecipitates were collected by protein G-Sepharose beads (Oncogene Science, Inc.). The immunoprecipitates were washed four times gently with washing buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10% glycerol, 0.1% Triton X, 100 mM sodium fluoride, and 2 mM sodium orthovanadate) before analysis.

Metabolic Labeling with [^{35}S]Methionine. Growth factor–starved cells were washed and resuspended at 10^7 cells/ml in methionine-free medium with 150 μCi ml^{-1} [^{35}S]methionine (Amersham) for 6 h. Radiolabeled cells were stimulated with growth factors and immediately lysed in lysis buffer as described above. After removing insolved material by centrifugation, cell lysates were used for immunoprecipitation.

Results

Coimmunoprecipitation of Shc Protein with p210bcr-abl in Three Different BCR-ABL Transfected Cell Lines. The three different BCR-ABL transformed cell lines, bcr-abl32DC13, H7bcr-ablA54, and MMTVras.bcr-ablG7 were factor independent for proliferation (data not shown). When compared with the parental cell lines, BCR-ABL transformed cell lines exhibited prominent tyrosine phosphorylation of proteins as determined by antiphosphotyrosine immunoblotting (Fig. 1 *A*). These BCR-ABL transformed cell lines were also shown to express p210bcr-abl by anti-ABL immunoblotting after anti-BCR immunoprecipitation (Fig. 1 *B*).

To analyze the interaction of Shc with other proteins in our cell lines, it was first established that all cell lines equally expressed Shc p46/p52 proteins (Fig. 2 *A*). Because Shc proteins possess a COOH-terminal SH2 domain, but do not possess any obvious catalytic domain (27), the possibility was considered that they might interact with other cytoplasmic signaling proteins. To test this hypothesis, the fate of Shc proteins in BCR-ABL transformed cells was examined. Cells were lysed and incubated with anti-Shc Abs, and the precipitated proteins were analyzed by immunoblotting with antiphosphotyrosine Abs (Fig. 2 *B*). Shc proteins (p46Shc and p52Shc) were phosphorylated on tyrosine in the BCR-ABL transformed cells compared with parental cell lines (Fig. 2 *B*). The background band around 50 kD is the Ig H chain from the immunoprecipitation. Since other proteins phosphorylated on tyrosine including Shc itself (approximate molecular weights of 210, 154, and 120) were coimmunoprecipitated with Shc proteins in the BCR-ABL, transformed cells, we investigated the possibility that p210bcr-abl was associated with Shc proteins. To test for complex formation, cell lysates were immunoprecipitated with anti-Shc or control Abs followed by SDS-PAGE and immunoblotting with an anti-ABL Ab (Fig. 2 *C*). The anti-Shc immune complexes contained

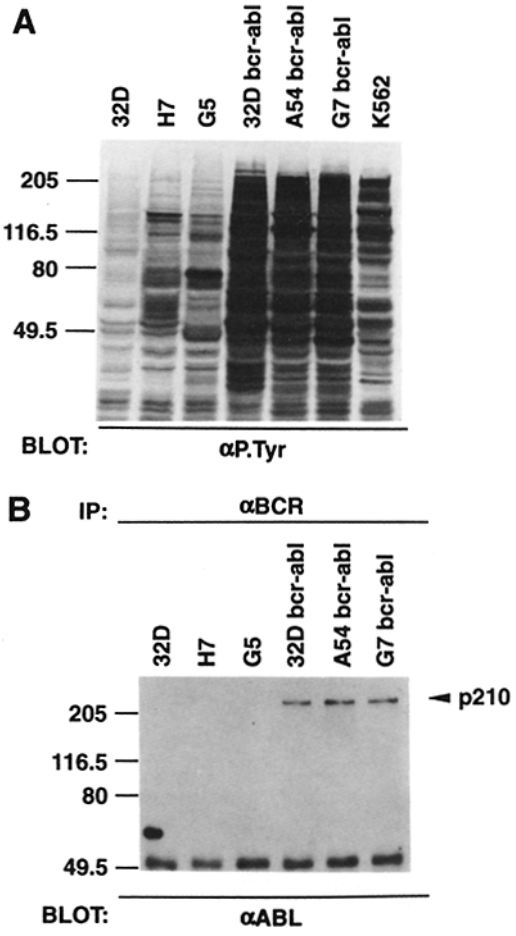


Figure 1. Expression of p210bcr-abl in three different BCR-ABL transfected cell lines. The parent cell lines are: 32DC13 (*32D*), NFS/N.1 H7 (*H7*), MMTVras.G5 (*G5*). The pGD210 transformed cell lines are: bcr-abl32DC13 (*32Dbcr-abl*), H7bcr-abl A54 (*A54bcr-abl*), MMTVras.bcr-abl G7 (*G7bcr-abl*). (*A*) Whole cell lysates from each cell line were prepared and 50 μg of total protein analyzed by 7.5% SDS-polyacrylamide gel, immunoblotted with antiphosphotyrosine Ab (4G10). (*B*) Cell lysates from the indicated cell lines were immunoprecipitated (*IP*) with anti-BCR, and immunoblotted (*BLOT*) with anti-ABL mAb. Molecular weight markers are in kilodaltons.

the p210bcr-abl protein that reacted with anti-ABL Ab (Fig. 2 *C*).

Because the anti-Shc Ab used in these studies was prepared against a region of Shc containing an SH2 domain, it was possible that the anti-Shc Ab precipitated p210bcr-abl by reacting with an ABL SH2 domain. To further examine the possibility that Shc and p210bcr-abl could be specifically coimmunoprecipitated, cell lysates were immunoprecipitated with a specific anti-ABL Ab (directed against the unique COOH-terminal domain of ABL) and immunoblotted with anti-Shc (Fig. 2 *D*). Shc proteins were detected in anti-ABL immunoprecipitates from the BCR-ABL transformed cells, but not from the parental cell lines (Fig. 2 *D*).

Coimmunoprecipitation of Grb2 Proteins with p210bcr-abl in BCR-ABL Transformed Cells. It was first established that

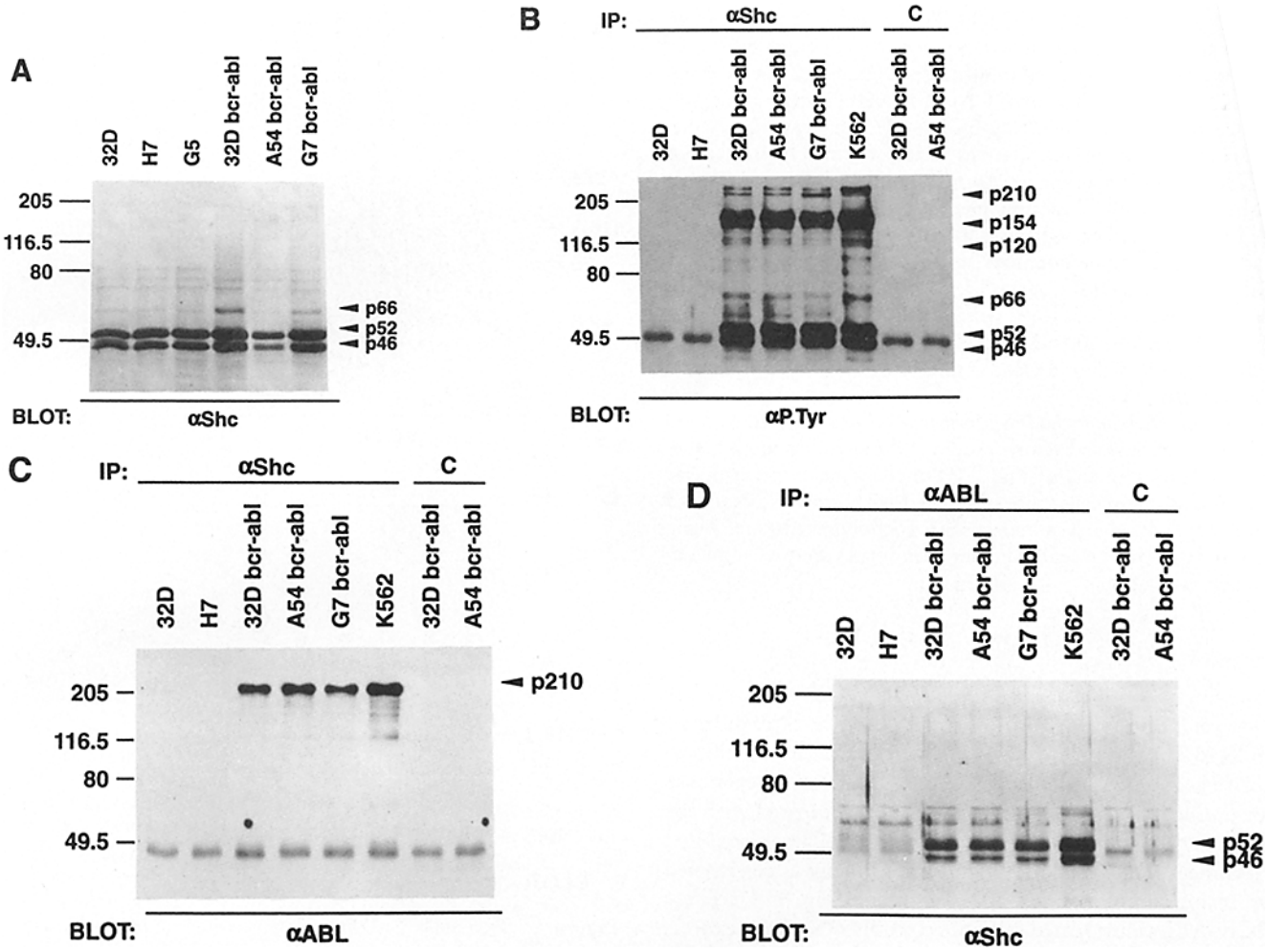
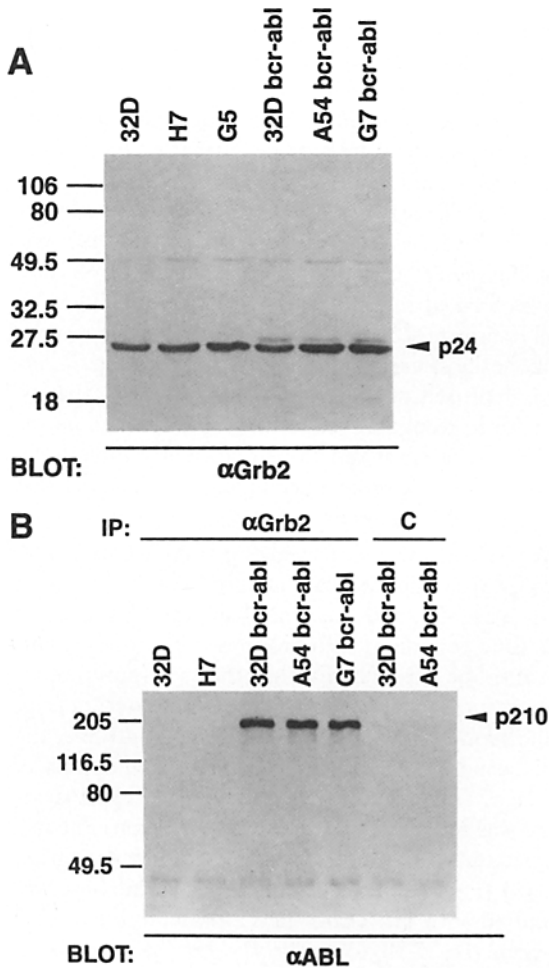


Figure 2. Formation of a p210bcr-abl–Shc complex in BCR-ABL transfected cell lines. (*A*) Whole cell lysates from each cell line were prepared, and 20 μg of total protein analyzed by 7.5% SDS-polyacrylamide gel, immunoblotted with anti-Shc mAbs. (*B*) Cell lysates from the indicated cell lines were immunoprecipitated (*IP*) with affinity-purified anti-Shc or nonimmune rabbit Ig Abs as a control (*c*, as noted for immunoprecipitation), and immunoblotted (*BLOT*) with antiphosphotyrosine Abs (RC20). The background band around 50 kD is the Ig H chain from the immunoprecipitation. (*C*) Affinity-purified anti-Shc or control Ab immunoprecipitates from the indicated cell lines were probed with anti-ABL mAbs. (*D*) The same lysates were immunoprecipitated with anti-ABL mAbs and immunoblotted with anti-Shc.

all cell lines equally expressed Grb2 proteins from whole cell lysates (Fig. 3 *A*). To determine whether Grb2 might associate with p210bcr-abl, cell lysates in each cell line were immunoprecipitated with anti-Grb2 and blotted with anti-ABL. p210bcr-abl was detected in anti-Grb2 immunoprecipitates in the BCR-ABL transformed cell lines, but not parental cell lines (Fig. 3 *B*). Conversely, the cell lysates were immunoprecipitated with anti-ABL, and were immunoblotted with anti-Grb2. The anti-ABL immunocomplex contained Grb2 protein in the BCR-ABL transformed cell lines (Fig. 3 *C*).

Formation of a Shc–Grb2 Complex in BCR-ABL Transformed Cells. To investigate the condition under which Shc proteins physically interact with Grb2, cell lysates from parent cells or BCR-ABL transformed 32DC13 cells were immunoprecipitated with anti-Shc or anti-Grb2, and these immune complexes were then analyzed by immunoblotting with anti-Shc (Fig. 4, *left*). p46 Shc and p52 Shc could be readily detected in anti-Grb2 immunoprecipitates from BCR-ABL transformed 32DC13 cells but not from parent 32DC13 cells. In a similar experiment, Grb2 was detected in anti-Shc immunoprecipitates from BCR-ABL transformed 32DC13 cells (Fig. 4, *right*). These results demonstrate that p210bcr-abl forms a single, specific complex with Shc and Grb2. Identical results, in which p210bcr-abl expression led to formation of complexes containing Shc-Grb2, were found with p210bcr-abl transformed clones, H7bcr-abl.A54, and MMTVras. bcr-ablG7 (data not shown).

SLF Induces Tyrosine Phosphorylation of Shc in MMTVras.G5 Cells. Shc was previously shown to be phosphorylated on tyrosine residues in cells responding to epidermal growth factor (EGF), platelet-derived growth factor, and insulin (27, 32–34). Furthermore, activation of the EGF receptor has been shown to induce the formation of complexes containing the autophosphorylated EGF receptor, and Shc and Grb2 (29). We



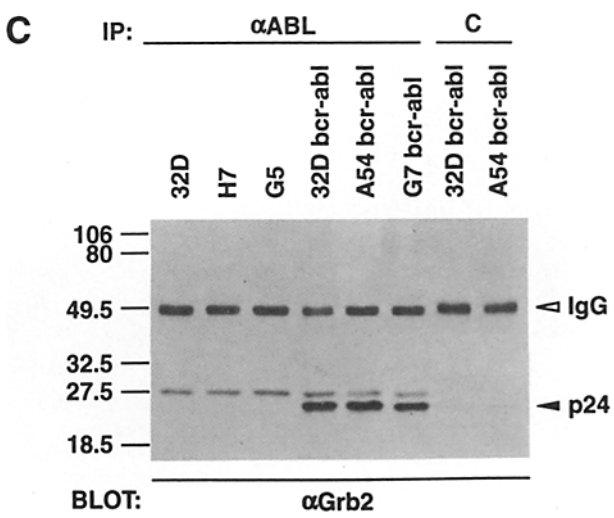


Figure 3. Formation of a p210bcr-abl–Grb2 complex in BCR-ABL transformed cell line. (*A*) Whole cell lysates from each cell line were analyzed by 10% SDS-polyacrylamide gel, immunoblotted with anti-Grb2 mAb. (*B*) Affinity-purified anti-Grb2 or nonimmune rabbit Ig immunoprecipitates from the indicated cell lines were immunoblotted with anti-ABL mAbs. (*C*) The same cell lysates were immunoprecipitated with anti-ABL mAbs or control mouse Igs and immunoblotted with anti-Grb2 mAb. The background bands around 50 and 27 kD are the Ig from immunoprecipitation.

therefore investigated whether Shc and Grb2 were potential targets of activated c-*kit* receptor.

In the factor-dependent MMTVras.G5 parental cell line, which expresses abundant c-*kit* receptors, stimulation of factor-starved cells with SLF led to an intense phosphotyrosine substrate band at 52 kD, putative Shc (Fig. 5). The substrate was most intensely tyrosine phosphorylated from 2 to 5 min after SLF stimulation. The cell lysates from MMTVras.G5 cells stimulated with SLF for 5 min were immunoprecipitated with anti-*kit* or anti-Shc Abs. The immunoprecipitates were resolved by 7.5% SDS-PAGE and were visualized by immunoblotting with antiphosphotyrosine (Fig. 6).

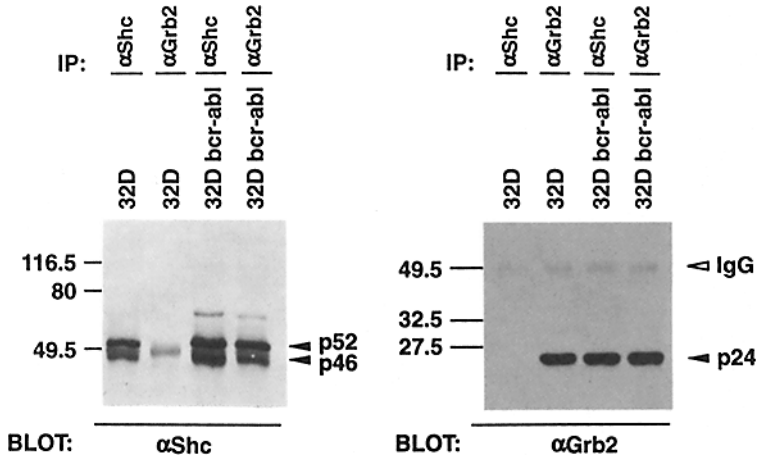


Figure 4. Coimmunoprecipitation of Shc and Grb2 in BCR-ABL transformed cell line. Cell lysates prepared from parental 32DC13 (*32D*) or transformed bcr-abl 32DC13 (*32Dbcr-abl*) were immunoprecipitated with affinity-purified anti-Shc and anti-Grb2. The immunoprecipitates were subjected to immunoblot analysis with anti-Shc mAb and anti-Grb2 mAb.

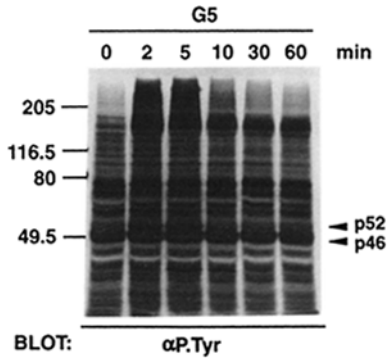


Figure 5. Time dependence of SLF-induced tyrosine phosphorylation in MMTVras.G5 cells. MMTVras.G5 cells (10^7/ml) were stimulated with SLF (50 ng/ml) for the indicated times. Cell lysates were analyzed by 7.5% SDS-PAGE and immunoblotted with antiphosphotyrosine (4G10).

Immunoprecipitated c-*kit* protein was strongly tyrosine phosphorylated in response to SLF in MMTVras.G5 cells, confirming the identity of the 145–160-kD phosphotyrosine substrate in whole cell lysates of SLF-stimulated cells (Fig. 6 *A*, lanes *1* and *2*). In these lanes, it was apparent that the background bands at 52 kD were the Ig H chain from control IgG immunoprecipitation (see below and Fig. 6 *B*, lane *1–3*). Stimulation of MMTVras.G5 cells with SLF induced a prominent tyrosine phosphorylation of p46 Shc and p52 Shc proteins revealed in the lysates immunoprecipitated with anti-Shc (Fig. 6 *A*, lanes *3* and *4*). In anti-Shc immunoprecipitates, SLF had also induced tyrosine phosphorylation of a fainter 145–160-kD protein in MMTVras.G5 cells which corresponds to the size of the immunoprecipitated c-*kit* receptor in these cells (Fig. 6 *A*, lanes *3* and *4*).

To determine whether Shc might associate with c-*kit* receptor, cell lysates from MMTVras.G5 cells stimulated with SLF were immunoprecipitated with anti-*kit* or control rat IgG and then immunoblotted with anti-Shc Ab (Fig. 6 *B*, lanes *1–3*). Shc could not be detected in anti-*kit* immunoprecipitates upon SLF stimulation (Fig. 6 *B*, lanes *1–3*). On the other hand, Shc proteins were detected from anti-ABL immunoprecipitates in bcr-abl transformed MMTVras.bcr-abl.G7 cells (Fig. 6 *B*, lanes *4–6*). As noted above, the ~52-kD band in the lanes containing lysates subjected to anti-c-*kit* immunoprecipitation were not Shc, but rather Ig H chain (Fig. 6 *A*, lanes *1* and *2* vs. Fig. 6 *B*, lanes *1–3*).

SLF Induces the Formation of a c-kit–Grb2 Complex in Parental Cells. Shc Binds Stoichiometrically to Grb2 in Parental and p210bcr-abl Transformed Cell Lines. To obtain an overview of complexes formed in parental MMTVras.G5 cells or in p210bcr-abl transformed MMTVras.bcr-ablG7 cells, these cells were metabolically labeled with [^{35}S]methionine (Fig. 7 *A*). In part, this approach was necessitated by the poor capacity of the c-*kit* Ab to recognize reduced and denatured c-*kit* on Western blots, whereas it functioned efficiently for immunoprecipitation of native protein before reduction and denaturation (Figs. 6 and 7 *A*).

MMTVras.G5 cells were deprived of growth factor and labeled with [^{35}S]methionine. An aliquot was subjected to stimulation with SLF, and the other half was unstimulated (Fig. 7 *A*). One portion of each group was immunoprecipitated with anti-Shc Abs, whereas the other was immunoprecipitated with anti-c-*kit* or a nonimmune control serum (Fig. 7 *A*). In the groups immunoprecipitated with anti-Shc, the Shc p46/p52 bands were quantitatively unchanged upon SLF treatment (Fig. 7 *A*). On the other hand, after SLF treatment, there was a quantitative increase in these anti-Shc immunoprecipitates of ^{35}S proteins corresponding to the p160 band and p24 (Fig. 7 *A*). Subsequently, the same blot was immunoblotted with anti-Grb2. This 24-kD protein reacted with anti-Grb2 (Fig. 7 *B*). Conversely, when the lysates were immunoprecipitated with the anti-c-*kit*, there was evidence of an increase of p24 Grb2 in the complexes of SLF-treated cells (Fig. 7 *B*). In the same condition, Shc could not be detected in anti-c-*kit* immunoprecipitates upon SLF treatment (Fig. 6 *B*, lanes *1–3*).

In contrast to dependence on SLF treatment for Grb2 coimmunoprecipitation with Shc in parental MMTVras.G5 cells, in p210bcr-abl transformed derivative MMTVras.bcr-ablG7

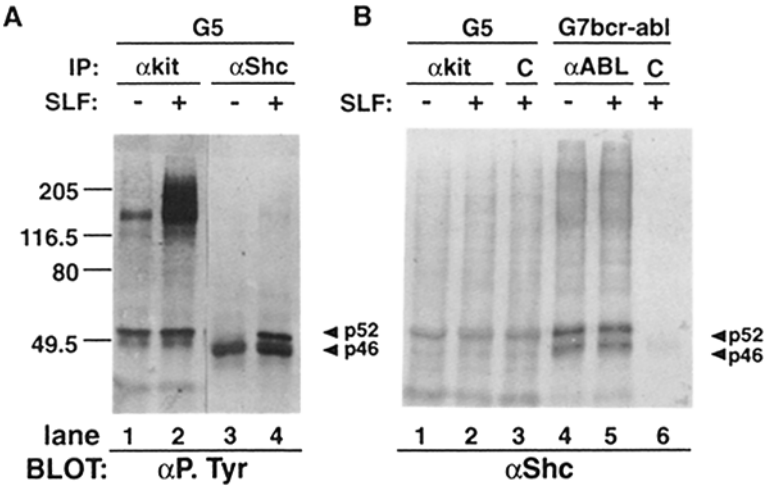


Figure 6. Tyrosine phosphorylation of c-*kit* and Shc proteins in response to SLF. Shc proteins were not immunoprecipitated with c-*kit*. (*A*) MMTVras.G5 cells were treated with SLF for 5 min before cell lysis. Cell lysates were immunoprecipitated with anti-kit or affinity-purified anti-Shc. Immunoprecipitates were separated by 7.5% SDS-PAGE and immunoblotted with antiphosphotyrosine (4G10). (*B*) MMTVras.G5 cells and MMTV ras. G7 cells were treated with SLF for 5 min before cell lysis. Cell lysates from MMTVras.G5 cells were immunoprecipitated with anti-*kit* or control rat IgG, and then immunoblotted with anti-Shc (lanes *1–3*). Cell lysates from MMTV.ras.bcr-ablG7 cells were immunoprecipitated with anti-ABL or control mouse IgG, then immunoblotted with anti-Shc (lanes *4–6*).

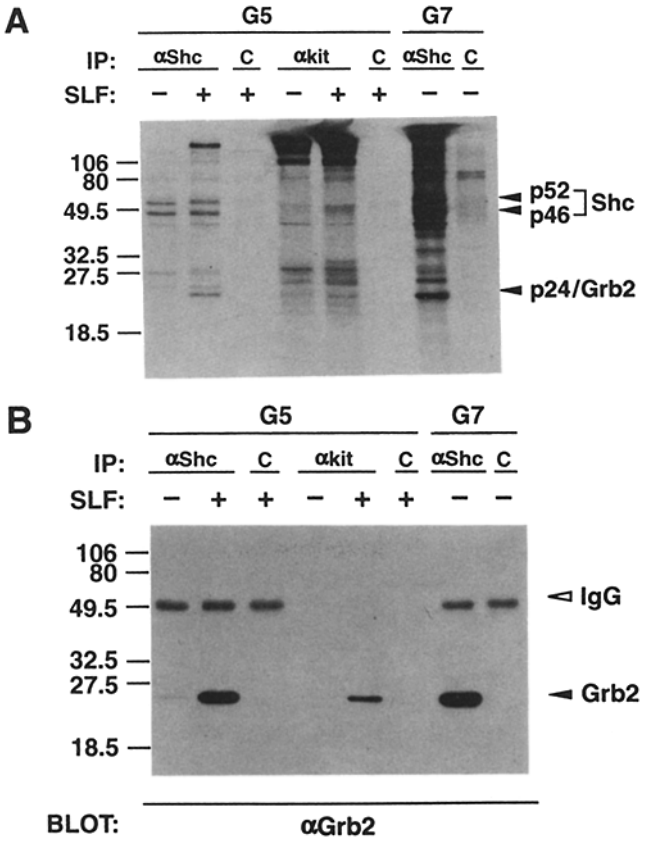


Figure 7. SLF-dependent association of Grb2 with c-*kit* in MMTVras.G5 cells. (*A*) MMTVras.G5 cells and MMTVras.G7 cells were metabolically labeled with [^{35}S]methionine for 6 h. After treatment with SLF for 5 min, the cell lysates were immunoprecipitated with anti-c-*kit* or affinity-purified anti-Shc or control rabbit Ig. Immunoprecipitates were separated by 10% SDS-PAGE and were visualized by autoradiography. (*B*) The same blot was immunoblotted with anti-Grb2 mAb.

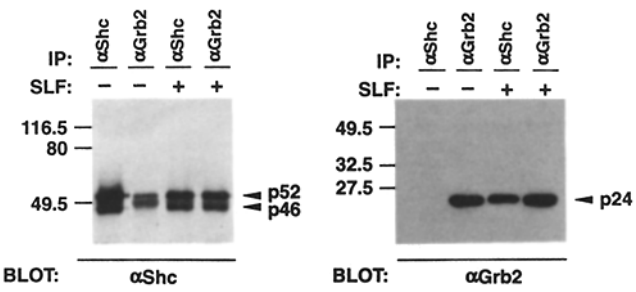


Figure 8. SLF induces formation of a Shc–Grb2 complex in MMTVras.G5 cells. MMTVras.G5 cells were treated with SLF for 5 min before cell lysis. Cell lysates were immunoprecipitated with affinity-purified anti-Shc and anti-Grb2, then immunoblotted with anti-Shc mAb and anti-Grb2 mAb.

cells, Grb2 was highly associated with Shc in the absence of SLF (Fig. 7, *A* and *B*). MMTVras.bcr-ablG7 cells were found to possess c-*kit* receptors by immunofluorescence after labeling with biotin-conjugated SLF and fluoresceinated avidin, and by the same metabolic labeling procedure, followed by c-*kit* immunoprecipitation, as described above (data not shown). However, the number of c-*kit* receptors were diminished compared to parental cells.

Finally, we examined SLF-induced formation of Shc–Grb2 complexes in MMTVras.G5 cells by developing duplicate blots with anti-Shc Ab or with the Grb2 Ab (Fig. 8). After SLF treatment, coimmunoprecipitation of one species (Shc or Grb2) with the Ab against its binding partner was as efficient as direct immunoprecipitation using self-specific Abs (Fig. 8). This data argues for high affinity binding occurring between Grb2 and Shc after SLF treatment. However, only Grb2 appears to tightly complex c-*kit* upon SLF treatment (Fig. 7). In control experiments, we addressed the possibility that Grb2 might be tyrosine phosphorylated in its interaction with c-*kit*. We found no evidence for Grb2 tyrosine phosphorylation after SLF treatment (data not shown).

Discussion

A dominant negative inhibitory ras mutant (Asn17) reversed transformation caused by nonreceptor tyrosine kinase oncogenes in fibroblasts (13). This observation suggested there might be a role for p21ras in hematopoietic signaling by the nonreceptor tyrosine kinase oncogene, p210bcr-abl. In fact, it was recently shown that p210bcr-abl effects p21ras activation, evidenced by GTP loading, in hematopoietic cell lines transformed by p210bcr-abl (14).

In constructing an hypothesis to understand how p210bcr-abl might couple to p21ras activation, we considered the possible role of "adaptor" proteins Shc and Grb2. She was identified in fibroblasts as a transforming protein, whose structure is lacking in obvious catalytic domains (27). However, Shc is a tyrosine kinase substrate and itself contains an SH2 domain capable of binding phosphotyrosine (27, 28). Shc can also induce PC12 pheochromocytoma cells to differentiate. Shc induction of PC12 differentiation is blocked by the dominant negative (Asn17) ras, indicating that Shc functions upstream of p21ras (29).

Grb2 is another structurally simple adaptor protein that consists entirely of an SH2 domain and two SH3 domains (20). It is not a tyrosine kinase substrate and it binds avidly to autophosphorylated kinase receptors (20). It has a proximate role in coupling to p21ras activation because of the binding of its SH3 domains to the p21ras guanine nucleotide exchange factor, Sos (20–23, 25, 26). This binding interaction is independent of external signals (22). In contrast, association of Grb2 with Shc only occurs when Shc has been tyrosine phosphorylated (29, 34).

In this study, we compared the coupling of these adaptor proteins, Shc and Grb2, with p210bcr-abl vs. c-*kit*. We performed this study to gain understanding of the different requirements for coupling with the p21ras pathway by these different classes of tyrosine kinase hematopoietic effectors (receptors vs. nonreceptors). In factor-starved nontransformed parental MMTVrasG5 or 32DC13 cells, Shc and Grb2 proteins were not associated (Figs. 4, 7, and 8). However upon treatment of the SLF-dependent cell line MMTVras.G5 with SLF, strong association between Shc and Grb2 occurred (Figs. 7 and 8). This association was of such an affinity that stoichiometric recovery of either species could be achieved by immu-

noprecipitation of cell lysates with an Ab against its partner or against self (Fig. 8). This was determined by developing duplicate Western blots with Abs against Shc or Grb2 (Fig. 8).

On the other hand, in three different p210bcr-abl transformed cells, Grb2 and Shc were constitutively associated in serum-starved cells lacking specific growth factors (IL-3 or SLF) (Figs. 4 and 7). In these cells expressing p210bcr-abl, Shc proteins were heavily tyrosine phosphorylated (Fig. 2 *B*). The constitutive tight association of Shc and Grb2 in p210bcr-abl transformed cells involved the presence of p210bcr-abl in the same complex (Fig. 2, *C* and *D*, and Fig. 3, *B* and *C*). Within this complex, it therefore would appear Shc is a direct substrate of p210bcr-abl. In such a model, Shc may act as a "bridge" or "spacer" for coupling p210bcr-abl to the guanine nucleotide exchange factor (Sos) through Grb2. However, it is also possible that Grb2 will bind directly to tyrosine phosphorylated p210bcr-abl. Further experiments are required to determine whether direct or indirect (via Shc) binding of Grb2-Sos to p210bcr-abl may be preferred for ras activation.

Although several biochemical arguments favor the possibility that coupling of tyrosine kinases to p21ras activation should involve enhanced guanine nucleotide exchange rate, our data provide a physical link by which such a process could be facilitated. However, our data do not rule out the possible additional involvement of p210rasGAP in upstream regulation of p21ras by p210bcr-abl. Druker et al. (35) previously demonstrated that p120rasGAP exists in a tight complex with p210bcr-abl. Maintenance of the GTP bound state of p21ras may involve the peculiar spacing within these complexes or an allosteric conformation of p120rasGAP, so that rasGAP might be unavailable to catalyze GTP hydrolysis on p21ras. It has been demonstrated that tyrosine phosphorylation of p120rasGAP does not alone negatively affect p21ras activity (36, 37). Both features of this model (involvement of Grb2-Sos and p120rasGAP) would require proximity of p210bcr-abl–Grb2-Sos to the integral membrane ras protein. The capacity for F-actin binding may provide for that function in p210bcr-abl in the absence of a myristoylation motif that characterizes v-abl (38).

In contrast to the high affinity interaction of both Shc and Grb2 with p210bcr-abl, only Grb2 appeared to bind with high affinity with the activated c-*kit* receptor (Fig. 7). We could not detect the direct association between activated c-*kit* receptor and Shc proteins (Fig. 7). Thus, even if Shc proteins directly associated with c-*kit* receptor, the association is very low affinity. Alternatively, tyrosine phosphorylation of Shc might be an indirect step mediated by a nonreceptor tyrosine kinase that binds directly to c-*kit*. Further work to unravel the features of c-*kit* signaling to p21ras is warranted.

In summary, this report provides an attractive explanation of the mechanism for p210bcr-abl signaling in the p21ras pathway. By exerting its constitutive tyrosine kinase activity in a complex with Shc and Grb2, it appears likely that an avenue is opened for coupling p210bcr-abl with a growth factor–independent p21ras pathway via enhanced guanine nucleotide exchange. This pathway used by p210bcr-abl appears to differ from the similar pathway used by the tyrosine kinase receptor c-*kit*, both in terms of the requirements for specific ligand activation and in the signaling molecules that avidly bind to the tyrosine kinase effector. However, both p210bcr-abl and activated c-*kit* have the capacity for inducing high affinity association between Grb2 and Shc.

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