In Vivo Association of v-Abl with Shc Mediated by a Non-phosphotyrosine-dependent SH2 Interaction*

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A necessary downstream element of Abelson murine leukemia virus (Ab-MLV)-mediated transformation is Ras, which can be activated by the phosphotyrosine-dependent association of Shc with the Grb2-mSos complex. Here we show that Shc is tyrosine-phosphorylated and associates with Grb2 in v-Abl-transformed cells, whereas Shc in NIH3T3 cells is phosphorylated solely on serine and is not Grb2-associated. In addition, Shc co-precipitates with P120 v-Abl and P70 v-Abl, which lacks the carboxyl terminus. Surprisingly, a kinase-defective mutant of P120 also binds Shc, demonstrating that Shc/v-Abl association is a phosphotyrosine-independent interaction. Glutathione S-transferase fusion proteins were used to map the interacting domains and showed that Shc from both NIH3T3 and v-Abl-transformed cells binds to the Abl SH2 domain and that P120 v-Abl binds to a region in the amino terminus of Shc. Consistent with these data, a v-Abl mutant encoding only the Gag terminal domain of the c-Abl non-receptor tyrosine kinase (1). In contrast to the tightly regulated c-Abl kinase (2, 3), v-Abl has constitutive tyrosine kinase activity, a feature required for transformation (1). Signaling via the Ras pathway is an essential part of the mechanism by which v-Abl induces transformation (4, 5). Expression of a dominant negative Ras (4) or microinjection of anti-Ras antibodies (5) causes a reversion of the Ab-MLV-transformed phenotype. Transformation by the Bcr-Abl chimeric protein, another oncogenic form of c-Abl, found in Philadelphia chromosome-positive chronic myelogenous leukemia (1, 6), is also blocked by dominant negative Ras (4).

The mechanism by which v-Abl activates Ras has not been elucidated. The Bcr-Abl protein may signal Ras via direct interaction with the Grb2 protein (7), a molecule that binds the guanine nucleotide exchange factor, mSos (8, 9). Localization of the Grb2-mSos complex to the plasma membrane activates Ras (9, 10). Bcr-Abl-Grb2 interaction involves sequences in the Bcr domain (7, 11, 12). This pathway is probably not used by v-Abl because the v-Abl protein does not contain a motif similar to that involved in Bcr-Grb2 interaction.

An alternative link to Ras, important in signaling by a large number of receptor tyrosine kinases including EGFR, Ins-R, PDGFR, c-Kit, c-ErbB2, c-ErbB3, c-RET (13, 14, 15, 16, 17, 18), the non-receptor tyrosine kinases, Src, Fps, FAK, Lyn, Syk (19, 20, 21), and the oncprotein polyoma middle T (22, 23), involves tyrosine phosphorylation of the Shc adaptor protein. Interactions between Shc and receptor tyrosine kinases occur when the receptors are activated by interaction with ligand; the autophosphorylation sites on the receptors provide binding sites for the Shc SH2 or amino-terminal domains (16, 24–26). Subsequent tyrosine phosphorylation of the YYNV motif in Shc by the receptors or their associated kinases creates a binding site for the Grb2 SH2 domain. Shc then associates with Grb2 which brings mSos into the complex (27–29). Such an interaction may be involved in factor independent growth of v-Abl-expressing mast cells (30). However, association with Grb2 and evidence of stimulation of signals downstream of Ras have not been reported in that system.

Because v-Abl lacks the Grb-2 binding site found in Bcr-Abl, we investigated the possibility that Shc protein associates with v-Abl and Grb-2 in v-Abl-transformed cells. These experiments demonstrate that Shc is tyrosine-phosphorylated in Ab-MLV-transformed fibroblasts and binds to Grb2. This interaction involves an unusual non-phosphotyrosine-dependent interaction that occurs between the v-Abl SH2 domain and the Shc amino terminus. The ability of Shc to bind Grb2 and the v-Abl SH2 simultaneously suggests a model for Shc-mediated Ras activation in Ab-MLV transformation similar to that of receptor tyrosine kinases.

EXPERIMENTAL PROCEDURES

Cells and Viruses—NIH3T3 fibroblasts and v-Abl-transformed NIH3T3 cells were grown in DMEM (Life Technologies, Inc.) supplemented to contain 10% Cosmic Calf serum (HyClone), 2 mM l-glutamine, and 50 μg/ml gentamycin (Life Technologies, Inc.). The NIH3T3 cell lines ANN-1, transformed with Ab-MLV-P120 strain (31), and 70wt, transformed with Ab-MLV-P70 (32), were described previously. Virus was obtained either by transfection of NIH3T3 cells using the calcium phosphate technique (33) followed by superinfection with Moloney murine leukemia virus (Mo-MLV) (34) or by transfection of 293T cells as described previously (35). In some cases, NIH3T3 cells were infected and grown in 0.8 mg/ml G418 (Life Technologies, Inc.) to select for cells expressing the neomycin resistance marker within the virus.

v-Abl Mutants—The P120 v-Abl kinase domain mutant (P120k-) was created by site-directed mutagenesis in M13 phage using the modified method of Zoller and Smith described in Ref. 33. A mismatched primer

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1 The abbreviations used are: Ab-MLV, Abelson murine leukemia virus; Mo-MLV, Moloney murine leukemia virus; SH2, src homology region 2; SH1, src homology region 1; GST, glutathione S-transferase; DMEM, Dulbecco's modified Eagle's medium; RGG, rabbit γ-globulin; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.

back to top
RESULTS

Shc Is Tyrosine-phosphorylated and Associates with Grb2 in Ab-MLV-transformed Cells—To determine whether Shc is tyrosine-phosphorylated in Ab-MLV-transformed cells, lysates from ANN-1 cells, a P120 v-Abl-transformed NIH3T3 cell line, and uninfected NIH3T3 cells were precipitated with anti-Shc antibody and analyzed by Western blotting (Fig. 1B). Shc from ANN-1 cell lysates was highly phosphorylated while that recovered from NIH3T3 cells was not tyrosine-phosphorylated. The electrophoretic mobility of Shc differs between NIH3T3 and ANN-1 cell lysates (Fig. 1A). Other higher molecular weight phosphoproteins were also observed coprecipitating with Shc from ANN-1 cells (Fig. 1B). Shc from NIH3T3 cells migrates as three distinct bands, but Shc from ANN-1 cells migrates as several subspecies. A similar broadening of Shc bands has been observed after the addition of PKC activators (42). One-dimensional phosphoamino acid analysis of immunoprecipitated Shc from [32P]orthophosphate-labeled cells (Fig. 2, A and B) showed the presence of phosphotyrosine and phosphoserine in ANN-1-derived Shc and only phosphoserine in Shc from NIH3T3 cells. In addition, an increase in the total amount of phosphoserine was also observed in Shc from ANN-1 cells.

To determine if the tyrosine-phosphorylated Shc was able to bind Grb2, the lower portion of the blot shown in Fig. 1A was probed with anti-Grb2 antibody (Fig. 1C). Shc from ANN-1 cells binds Grb2, in contrast to Shc precipitated from NIH3T3 cells which does not. Therefore, the presence of v-Abl causes the association of Shc and Grb2, most likely through the phosphorylation of Tyr-317 of Shc. This pattern is consistent with

replacing a G with an A at position 2070 within the kinase domain sequence of the Ab-MLV-P120 genome (36) resulted in a substitution of Asp for Asp at residue 484 of the P120 protein. The presence of the mutation was verified by dideoxy method sequencing with the Sequenase kit (Promega), and sequence containing the mutation was cloned into pANL2, a vector based on pABLSVneo (37). This vector is composed of pBS1 BamHI fragment containing the neo resistance gene, which is flanked by an SV40 promoter cloned into the BamHI site at position 4355. These sequences are flanked by two complete long terminal repeats. The GagSH2 v-abl mutant was constructed by amplifying bases 621–1685 of the Ab-MLV P120 genome by PCR from pUC120 (32). The 5' primer included an EcoRI site and the 3' end of the Gag-coding region. The 5' end of the Gag-coding region included the 3' end of the SH2 coding region, a termination codon, and an EcoRI site. The PCR product was cloned into the pSRα vector (35) at the EcoRI site, and the amplified sequence was verified by dideoxy method sequencing.

Antibodies—The monoclonal antibodies, H548, directed against p12 Gag determinants present in v-Abl (38); a gift of B. Chesebro, Rocky Mountain Laboratories, Hamilton, MT), 24–21, directed against an epitope in the v-Ab carbohydrate domain (39), and 19–84, directed against a v-Abl SH1 domain epitope (39), were used to detect v-Abl proteins. Anti-phosphotyrosine monoclonal antibodies were purchased from Upstate Biotechnology. Anti-Shc monoclonal and polyclonal antibodies as well as anti-Grb2 monoclonal antibodies were purchased from Transduction Laboratories. Anti-GST monoclonal antibodies were obtained from Pharmacia Biotech Inc. Anti-phosphotyrosine antibodies were purchased from Upstate Biotechnology. Anti-mouse and anti-rabbit IgG alkaline phosphatase-conjugated antibodies were purchased from Promega. Anti-mouse horseradish peroxidase-conjugated antibody was obtained from Amersham.

GST Fusion Proteins—The GST fusion proteins were all constructed in the pGEX-3X vector (Pharmacia Biotech Inc.) and PCR primers for the inserts contained either a BamHI or an EcoRI linker. For the GST-AbSH2SH1 construct, PCR was performed to amplify bases 1329–2441 of v-Ab P120 using pUC120. To create the GST-Ab SH1 and GST-Ab SH2 constructs, the v-Ab sequences, bases 1686–2441 and bases 1686–2441, respectively, were amplified from pUC120. The GST-Ab SH2 was constructed by amplifying bases coding for residues 146–251 of v-Ab from pGKX-4 (40). The GST-Arg SH2 construct was a gift of G. Kruh (Fox Chase Cancer Center, Philadelphia, PA), and the GST-Shc construct was a kind gift of B. Schaffhausen (Tufts University, Boston, MA). Anti-mouse and anti-rabbit IgG alkaline phosphatase-conjugated antibodies were purchased from Promega. Anti-mouse horseradish peroxidase-conjugated antibody was obtained from Amersham.
Shc-mediated Ras activation as seen with other tyrosine kinases (16, 18, 20, 29). Non-Tyr(P)-mediated v-Abl/Shc Interaction

Most interactions of Shc with tyrosine kinases have shown that binding of Shc requires a tyrosine-phosphorylated motif (14, 17, 24, 25, 28). To assess the contribution of v-Abl kinase activity to v-Abl/Shc association, NIH3T3 cells expressing the mutant Ab-MLV-P120k-\(^{-}\), which encodes a kinase-defective P120 v-Abl, were utilized. P120k-\(^{-}\) contains an Asp to Asn amino acid change in the kinase domain rendering it kinase-defective. Cells expressing this protein do not contain increased levels of phosphotyrosine (Fig. 4, A and C) and immunoprecipitated P120k-\(^{-}\) has no detectable kinase activity in vitro (data not shown). P120k-\(^{-}\) was immunoprecipitated from cells ex-
pressing the mutant virus and analyzed on Western blots for Shc coprecipitation (Fig. 4, B and D). Immunoprecipitation of P120\(^{\text{v-Abl}}\) showed that Shc still bound to v-Abl. Therefore, Shc binding is independent of v-Abl kinase activity.

**v-Abl-Shc Interaction Involves the v-Abl SH2 Domain**—To identify the domains by which v-Abl associates with Shc, GST fusions of the Abl SH1 and SH2 domains were made and bound to glutathione-Sepharose beads to precipitate cell lysates for Western analysis (Fig. 5A). Shc was precipitated from both NIH3T3 and ANN-1 cell lysates by GST-Abl SH2 and by a GST fusion consisting of both the Abl SH1 and SH2 domains. Shc did not precipitate with the GST-Abl SH1 fusion protein. Therefore, the Abl SH2 domain alone is sufficient for Shc binding. Furthermore, the ability of the Abl SH2 domain to bind Shc from NIH3T3 cell lysates shows that neither v-Abl kinase activity nor Shc tyrosine phosphorylation is required for this interaction. The amounts of Shc recovered with the GST-Abl SH2SH1 fusion are somewhat lower than might be expected based on the results obtained with the GST-Abl SH2. Because the GST-Abl SH2SH1 protein is not kinase-active in vitro (data not shown), this difference is not mediated by phosphorylation of Shc by the fusion protein. However, the presence of SH1 sequences may alter the interaction between Abl SH2 and Shc in some fashion. Although the amount of Shc binding to the GST-Abl SH2 differs between NIH3T3 and ANN-1 cell lysates (Fig. 5A), this difference is less prominent in additional experiments (data not shown). GST fusion precipitation experiments also showed that a GST-Arg (Abl-related gene) SH2 domain binds Shc as well (Fig. 5E). The Arg SH2 domain differs from Abl by only 10 residues. However, consistent with previously published results (44), a GST-Src SH2 fusion did not bind Shc under our conditions (Fig. 5E). This suggests that the in vitro binding of Shc in our system is not a general property of SH2 domains.

A mutant Ab-MLV was made to test whether the Abl SH2 domain is sufficient for interaction in vivo. The GagSH2 virus, which consists of the Ab-MLV Gag-coding region and the Abl SH2-coding region followed by a stop codon, was expressed in NIH3T3 cells. Immunoprecipitates of the GagSH2 protein were analyzed by Western blotting with anti-Shc antibodies, revealing that Shc coprecipitated with the GagSH2 protein (Fig. 5, C and D). The Abl SH2 domain is therefore sufficient for binding of Shc both in vivo and in vitro. To examine whether the binding is direct or requires an intermediary for complex formation, the ability of the Abl SH2 to bind purified Shc was tested. Affinity-purified GST-Shc was treated with Factor Xa to release a full-length Shc protein. The GST-Abl SH2 was able to precipitate the Shc protein as observed by Western analysis with anti-Shc antibodies (Fig. 5F) despite the bacterial origin of both proteins.

Western blot analysis of GST-Abl SH2 precipitates from NIH3T3 and ANN-1 cells with anti-Grb2 antibody showed that Grb2 was precipitated with the GST-Abl SH2 in ANN-1 lysates but not in NIH3T3 lysates (Fig. 5B). This result presumably reflects the association of Grb2 with tyrosine-phosphorylated Shc in ANN-1 cells. In NIH3T3 cells, Shc and Grb2 are not associated, and, therefore, Grb2 would not be precipitated by the GST-Abl SH2 protein. Accordingly, these data suggest that binding of Shc to the Abl SH2 domain does not interfere with binding of Grb2 to Shc at tyrosine 317 and that Shc possesses independent binding sites for Grb2 and v-Abl. The potential for a v-Abl-Shc-Grb2 complex to form in Ab-MLV-transformed cells is suggestive of the Shc-mediated Ras activation described for receptor protein tyrosine kinases.

**v-Abl Binds to the Amino Terminus of Shc**—GST fusion proteins of the three major domains of the Shc protein, the aminoterminus (NT; residues 1–232 of p52 Shc), the collagen-homology domain (CH; residues 233–377), and the Shc SH2 domain bind Shc as well (Fig. 5). The GagSH2 virus, which consists of the Ab-MLV Gag-coding region and the Abl SH2-coding region followed by a stop codon, was expressed in NIH3T3 cells. Immunoprecipitates of the GagSH2 protein were analyzed by Western blotting with anti-Shc antibodies, revealing that Shc coprecipitated with the GagSH2 protein (Fig. 5, C and D). The Abl SH2 domain is therefore sufficient for binding of Shc both in vivo and in vitro. To examine whether the binding is direct or requires an intermediary for complex formation, the ability of the Abl SH2 to bind purified Shc was tested. Affinity-purified GST-Shc was treated with Factor Xa to release a full-length Shc protein. The GST-Abl SH2 was able to precipitate the Shc protein as observed by Western analysis with anti-Shc antibodies (Fig. 5F) despite the bacterial origin of both proteins.

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domain (SH2; residues 378–473), were used to identify the region which binds v-Abl. The GST fusion proteins were bound to glutathione-Sepharose beads and used to precipitate ANN-1 cell lysates as described above. Western analysis of the precipitated was done using α-Abl antibody. Approximately equal amounts of GST fusion proteins were used in each precipitation with the exception of GST-NT1 and NT2 which had 30–40% less fusion protein than the GST-NT precipitation (data not shown). α-Abl, 24–21 antibody. C, GST and GST-Shc NT were used to precipitate 0.5 μg of Abl SH2SH1 domain cleaved from the bacterially generated fusion as described under “Experimental Procedures.” The precipitates were analyzed by Western blotting. αAbl, 19–84.

**DISCUSSION**

Our results show that Shc is tyrosine-phosphorylated and associated with Grb2 in v-Abl-transformed cells. This association could lead to activation of Ras, an event required for v-Abl-mediated transformation. In receptor activation models, association of Shc with an activated receptor enables the receptor or a related kinase to directly phosphorylate Tyr-317, the Grb2 binding site on Shc (28). This modification allows the Shc-Grb2-mSos complex to assemble at the membrane in proximity to Ras (9, 10, 29). The binding we have documented may allow v-Abl or an associated kinase to directly phosphorylate Shc, stimulating the formation of a Shc-Grb2-Sos complex. The Gag domain of v-Abl may also provide a plasma membrane localized platform on which the Shc-Grb2-Sos complex can assemble. Localization of this complex is a critical event for mSos function because addition of a membrane localization signal to Sos is itself sufficient to activate Ras (10).

Some tyrosine kinases can bypass Shc and activate Ras by binding the Grb2-mSos complex directly through the Grb2 SH2 domain (8, 9, 27). Bcr-Abl accomplishes this by binding Grb2 to a phosphotyrosine-containing motif within the Bcr portion of the protein (7, 11, 12). However, v-Abl lacks a similar Grb2 binding site. Another possible site for direct Grb2 interaction with v-Abl is located within the carboxyl terminus (45). The Grb2 SH3 domains bind a proline-rich motif in the Abl carboxyl terminus in vitro. The carboxyl terminus of v-Abl also contains proline-rich motifs that bind Crk and Nck (45, 46), proteins that can associate with the Ras activators, C3G and mSos (47–49). However, the efficient transformation of NIH3T3 cells by P70 (32), which lacks these proline-rich motifs, precludes any essential role for these interactions. Tyrosine phosphorylation of Shc and its association with Grb2 could allow Ras signaling in the absence of Nck, Crk, and Grb2 binding sites.

The v-Abl/Shc interaction we have documented occurs in a phosphotyrosine independent manner and contrasts to the conventional view of SH2-mediated interactions which involve recognition of a phosphotyrosine residue in the context of three residues carboxyl-terminal to the tyrosine (50). Tyrosine phosphorylation of v-Abl is not required because both the P120k⁺ and the GagSH2 proteins bind Shc. In addition, the GST-Shc SH2 fusion binds Shc and the GST-Shc NT binds Abl SH2SH1 in vitro; these proteins are produced in E. coli and do not contain phosphotyrosine (51, 52). The possibility that tyrosine phosphorylations, on Shc in vivo, mediate the interaction is excluded because Shc from NIH3T3 cells binds the GST-Shc SH2 fusion and phosphoamino acid analysis of Shc recovered from those cells shows no phosphotyrosine. Shc also binds to P120k⁻ and GagSH2 despite the absence of tyrosine phosphorylation observed on Western analysis (data not shown).

Recently, Owen-Lynch et al. (30) described the interaction of Shc with a v-Abl protein encoded by a temperature-sensitive (ts) strain of Ab-MLV at both the permissive and nonpermissive temperature. Previous descriptions of the ts strain used note the persistence of phosphotyrosine on v-Abl protein at the nonpermissive temperature (53). Our own experiments with another ts Ab-MLV yielded similar data (not shown). The persistence of phosphotyrosine at the nonpermissive temperature suggests that these ts systems may not be practical for assessing the phosphotyrosine independence of protein-protein interactions.

Although many SH2-mediated interactions involve phosphotyrosine, the number observed that occur independent of this modification is growing. Phosphotyrosine-independent association between the Ab-MLV and Bcr has been observed (54, 55). Two serine-rich regions of Bcr are important for this binding, but the specific motifs involved have not been identified. SH2 domains from other proteins including phospholipase Cγ, Src, and GTPase activating protein also bind to Bcr in this manner (54). The SH2 domain of the protein-tyrosine phosphatase SHPTP2 binds its own catalytic domain in the absence of phosphotyrosine (56). Another instance of phosphotyrosine-independent binding involves interactions of the Src and Fyn SH2
domains with Raf, a protein which is phosphorylated on serine residues (57). It is possible that the Abl/Shc interaction is mediated by phosphoserine; however, it has not been determined whether the GST fusion proteins are phosphorylated correctly. Serine and threonine phosphorylation has been observed on bacterially produced GST fusion proteins (51).

The interaction of v-Abl with the GST-Shc NT1 protein indicates residues 1–85 of p52 Shc are sufficient for binding. p46 Shc, which also binds v-Abl, possesses only residues 46–85 of that region. Therefore, the essential binding site must lie within the shared sequence of both p52 and p46. The region is also of interest because it contains the recently described phosphotyrosine binding domain (58). This domain binds to NXPxY motifs, including those found on polyoma middle T, EGFR, and Trk (23, 25). However, this property of the Shc amino terminus is not involved in v-Abl binding because the interaction is phosphorytose independent. Additionally, the GST-Shc NT1 and NT2 fusion proteins precipitate v-Abl, whereas similar GST fusions cannot bind NXPxY targets without the minimal 46–238 amino acid region (59).

Another interaction observed in the Shc amino terminus is the protein kinase C-dependent binding of the PEST-phosphatase to p52 Shc (42). In addition to inducing Shc/Grb2 association, the v-Abl interaction could also regulate the Shc signaling complex. The PEST-phosphatase binds to Shc but does not alter tyrosine phosphorylation of Shc or its association with Grb2 (42). Presumably, the PEST-phosphatase dephosphorylates other proteins in the Shc signaling complex. By binding to Shc, v-Abl could phosphorylate additional proteins in the complex. Furthermore, because the v-Abl and c-Abl SH2 domains are identical, Shc may be involved in cytoplasmic interactions that are important for normal c-Abl function.

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REFERENCES

1. Rosenberg, N. and Witte, O. N. (1986) Advances in Viral Research (Shatkin, A., ed) pp. 39–81, Academic Press, New York

2. Pendergast, A. M., Muller, A. J., Habib, T., Herrera, R., and Decker, S. J. (1994) J. Biol. Chem. 269, 3644–3648

3. Murtagh, K., Skladany, G., Haag, J., and Rosenberg, N. (1986) J. Biol. Chem. 261, 599–606

4. Habib, T., Pendergast, A. M., Pondel, M., Landau, N. R., Littman, D. R., and Witte, O. N. (1992) Mol. Cell. Biol. 12, 7185–7192

5. Wang, J. Y., and Baltimore, D. (1985) J. Virol. 57, 1182–1186
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