Rapid Communication

Tyrosine Phosphorylation of rasGAP and Associated Proteins in Chronic Myelogenous Leukemia Cell Lines

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The Philadelphia chromosome (Ph1), detected in virtually all cases of chronic myelogenous leukemia, is formed by a reciprocal translocation between chromosomes 9 and 22 that fuses BCR encoded sequences upstream of exon 2 of c-ABL. This oncogene produces a fusion protein (p210BCR/ABL) in which the ABL tyrosine kinase activity is elevated. This elevated kinase activity is essential for transformation, but the mechanisms involved are unknown. We report here that p210BCR/ABL kinase is phosphorylated on tyrosine in Ph1 (+) cell lines. Further, rasGAP coimmunoprecipitates with p210BCR/ABL in these cell lines. These results suggest that rasGAP or associated proteins are potential substrates for p210BCR/ABL kinase and thus directly link p210BCR/ABL with a signal transduction pathway known to be activated by hematopoietic growth factors (p210BCR/ABL).

Materials and Methods

Cells and cell culture. The 32Dc13 cell line was obtained from Joel Greenberger (University of Massachusetts Medical Center, Worcester). One subline of 32Dc13 expressing p210BCR/ABL and plasmids pGD and pGD210 were obtained from Richard van Etten and George Daley (MIT, Cambridge, MA). Four additional sublines expressing p210BCR/ABL were generated by transfection of plasmid pGD210 into 32Dc13 cells by electroporation as previously described7 using a BioRad Gene Pulsar (Richmond, CA) and selecting for 418 resistant sublines. All five of these cell lines were factor-independent and were shown to express p210BCR/ABL by immunoblotting as described below. Two 418-resistant cell lines were generated by transfection with pGD' vector alone. The 32Dc13, K562,11 BV173,12 NALM-1,13 NALM-6,14 U937,14 KG-1,15 and JOSK-M16 leukemic cell lines were cultured in RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum at 37°C, 5% CO2, WEHI-3 conditioned media, 15%, (as a source of IL-3) was added for 32Dc13 cells.

Immunoprecipitation, immunoblotting, and in vitro kinase assays. Cells were lysed in 1% NP40, 150 mmol/L NaCl, 20 mmol/L Tris pH 7.4, 10% glycerol containing 1 mmol/L phenylmethylsulfonylfluoride, 20 µg/mL aprotinin, and 1 mmol/L sodium orthovanadate at 10° cells/mL. Control experiments in which cells were lysed directly into boiling sample buffer containing 1% sodium dodecyl sulfate (SDS) indicated that lysis in 1% NP40 buffer extracted approximately 50% of the total p210BCR/ABL, consistent with previous studies indicating that a substantial fraction of p210BCR/ABL is bound to cytoskeletal proteins.7 One hundred microliters of each cellular NP40 lysate was immunoprecipitated with 5 µL rabbit anti-rasGAP antibodies (Upstate Biotechnology, Inc, Lake Placid, NY), raised against Trp E-GAP fusion protein containing amino acid residues 171 to 448 of human GAP), or 5 µL of two different nonimmune sera for 2 hours at 4°C. Immune complexes were collected by incubation with 30 µL protein A-Sepharose (Pharmacia, Uppsala, Sweden) for 30 minutes and then washed once with lysis buffer, twice with PBS, 250 mmol/L L-Tris pH 8.0, and once with phosphate-buffered saline (PBS). Samples were resuspended in 30 µL of PBS, 25 µL of 2x sample buffer was added, and samples boiled for 5 minutes.

Antiphosphotyrosine antibody immunoprecipitates were performed using 20 µL of 4G10 coupled to Protein A-Sepharose at 2 mg/mL with dimethylpiperimidine.19 Immunoaffinity columns using antiphosphotyrosine antibody 4G10 were performed as described.18 AntirasGAP immunoblots were blocked in 2% gelatin in Tris-buffered saline (TBS) for 1 hour at 25°C, then incubated for 2 hours at 25°C with rabbit anti-rasGAP antibody diluted 1:1,000 in TBS with 0.05% Tween 20 (BioRad). Immunobots were probed for with a monoclonal anti-ABL antibody 24-2l2O diluted 1:200 in TBS with 0.05% Tween 20 and developed as described.18 Anti-ABL immuno-
precipitates were performed for 2 hours at 4°C with anti-ABL antibody 24-21. Immune complexes were collected with Protein G-Sepharose. For immune complex kinase assays immunoprecipitates were performed as described above with the exception that the final wash was with kinase buffer (50 mmol/L Tris pH 7.4, 20 mmol/L MnCl2). A control immunoprecipitate of 32Dp210 clone 1 lysate was performed with a rabbit anti-polymyosin T-antigen antisera. Washed immunoprecipitates were resuspended in 40 μL kinase buffer supplemented with 10 μCi 32P (NEN, Boston, MA; 3,000 μCi/mmol) and incubated 20 minutes at 25°C. Samples were boiled for 5 minutes after the addition of 25 μL of 2x protein sample buffer and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was dried and exposed by autoradiography.

RESULTS

Expression of p210BCR/ABL in the murine 32Dc13 cell line is associated with the appearance of novel phosphotyrosine-containing proteins. Five independently derived factor-independent sublines of 32Dc13 expressing p210BCR/ABL were examined by anti-phosphotyrosine immunoblot before and after stimulation with IL-3. Figure 1 shows the results for clone 1; virtually identical results were obtained for the other four. When compared with the unstimulated parent cell line, the p210BCR/ABL (+) 32D cell lines exhibited prominent tyrosine phosphorylation of proteins of approximate molecular weight = 210, 190, 120, 93, 62, and 50 Kd. Increased tyrosine phosphorylation of several proteins, including a 93-Kd protein, was observed in the parent line after IL-3 stimulation as previously described. Anti-phosphotyrosine immunoblots of two control cell lines transfected with the pGD' vector alone were also examined. These cell lines were factor dependent, and the blots were indistinguishable from those shown for the 32Dc13 parent cell line shown in Fig 1 (data not shown).

Identification of tyrosine phosphorylation of p120 rasGAP. In preliminary experiments, it was noted that the band noted as p120 in the above experiments comigrated with rasGAP on one-dimensional gels. The possibility that the 120-Kd protein was rasGAP was investigated using an anti-rasGAP specific antisera. In the 32Dc13 cells, rasGAP was not detectably phosphorylated on tyrosine (Fig 2A and B). By contrast, rasGAP and the rasGAP-associated proteins p190 and p62 were phosphorylated on tyrosine in 32Dp210 cells (clone 1 is shown, similar results were obtained with each of the other four clones studied). As shown previously by Ellis et al, "p62" is composed of several bands in the 60- to 62-Kd range. Similar amounts of rasGAP were immunoprecipitated by anti-rasGAP sera from both lines (Fig 2C). RasGAP, p190, and p62 were also phosphorylated on tyrosine in the p210BCR/ABL (+) myeloid/erythroid cell line, K562, while p190 and p62, but not p120 rasGAP, were tyrosine phosphorylated in the lymphoid CML blast cell lines, BV-173 and NALM-1 (Fig 2A). Tyrosine phosphorylation of rasGAP or associated proteins was not detected in two Ph' (-) factor-independent myeloid cell lines (U937 and JOSK-M), a Ph' (-) pre-B cell ALL cell line (NALM-6), or in normal blood neutrophils from three donors or bone marrow mononuclear cells from two donors (Fig 2A, B, and data not shown).

Coimmunoprecipitation of rasGAP and p210BCR/ABL in 32Dp210 and K562 cells. In v-src transformed fibroblasts, tyrosine phosphorylated rasGAP reversibly associates with p60v-src, presumably through an interaction involving SH2 domains. As a tyrosine phosphorylated protein of M, 210 Kd was noted in anti-rasGAP immunoprecipitates of 32Dp210 and K562 cells, we asked whether rasGAP was associated with p210BCR/ABL. Cell lysates from 32Dp210 clone 1 or K562 were immunoprecipitated with anti-rasGAP or control antibody followed by SDS-PAGE and immunoblotting with an anti-ABL antibody (Fig 3A). In each case, the anti-rasGAP immune complexes contained a

![Fig 1. Expression of p210BCR/ABL in the murine 32Dc13 cell line is associated with the appearance of novel phosphotyrosine containing proteins. The parent cell line (32D) and a p210BCR/ABL (+) subclone were cultured overnight without IL-3, then stimulated with IL-3 (10% WEHI-3 conditioned medium) for 0 to 90 minutes. Phosphotyrosine-containing proteins were visualized by immunoblot. Virtually identical results were obtained with the other four p210BCR/ABL (+) sublines (data not shown).](image-url)
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NRS the anti-rasGAP antibody precipitated p210BCRIABL by reacting with an abl SH2 domain. Control experiments demonstrated that the anti-rasGAP antibody did not recognize p210BCRIABL by immunoblot; however, it remains possible that anti-rasGAP could immunoprecipitate p210BCRIABL. To further examine the possibility that rasGAP and p210BCRIABL can be specifically coimmunoprecipitated, cell lysates were immunoprecipitated with a specific anti-ABL antibody (directed against the unique carboxyterminal domain of ABL) and blotted with anti-rasGAP. rasGAP was detected in anti-ABL immunoprecipitates from 32Dp210 clone 1 cells, but not from the parent cell line (Fig 3B). The p210 band coimmunoprecipitated by anti-rasGAP was phosphorylated in an in vitro kinase assay, consistent with its identity as p210BCRIABL (Fig 3C). Also, the in vitro kinase products of an rasGAP immunoprecipitate were boiled, and reprecipitated by anti-ABL. The p210 band was immunoprecipitated, further indicating the identity of the p210 protein associated with rasGAP as p210BCRIABL (data not shown). Anti-rasGAP immune complexes obtained from three p210BCRIABL negative cell lines (32DcI3, KG-1, and U937) did not contain any proteins recognized by anti-ABL, nor did in vitro kinase reactions show bands at p210.

DISCUSSION

We show here that rasGAP and two associated proteins, p190 and p62,10 were heavily phosphorylated on tyrosine in a series of independently derived subclones of 32D cells expressing p210BCRIABL, and in several human Ph (++) cell lines. Further, p210BCRIABL, rasGAP, p62, and p190 were shown to coimmunoprecipitate from several of these lines, indicating that they likely existed as a complex in vivo. The domains of either rasGAP or p210BCRIABL that are responsible for this interaction have not yet been investigated. Because both rasGAP and p210BCRIABL have SH2 domains, it is possible that the interaction is mediated by SH2 domain interactions. The tyrosine phosphorylation of rasGAP and associated proteins was unique to p210BCRIABL (+) cells, and was not observed in the normal murine or human myeloid cells or other types of human leukemic cell lines examined in this study. In preliminary studies, we have also observed tyrosine phosphorylation of rasGAP, p190, or p62 in 8 of 10 cases of primary chronic myelogenous leukemia (CML) in blast crisis,21 indicating that the findings reported here are observed in primary CML cells as well as in cell lines that overexpress p210BCRIABL.

These results have several implications for understanding the pathogenesis of CML. Potential substrates for p210BCRIABL fusion protein tyrosine kinase have not previously been identified. Because rasGAP is both a regulator of p21ras function and also a potential downstream effector molecule for p21ras22 our data suggest that aberrant tyrosine phosphorylation of rasGAP could augment p21ras mediated signal transduction events. Because IL-3 and other hematopoietic growth factors activate p21ras23 the mitogenic signals initiated by these factors could be amplified in Ph (++) cells because of the tyrosine phosphorylation of rasGAP, p190, or p62 induced by p210BCRIABL. In
Fig 3. Coimmunoprecipitation of p210Bcr/Ab1 and rasGAP. (A) Anti-rasGAP or control antibody immunoprecipitates from the indicated cell lines were probed with anti-ABL antibodies. (B) Anti-ABL immunoprecipitates were probed with anti-rasGAP antibodies. (C) Immune complex kinase assay of 32Dc13 (lanes A and C) or a subclone (clone 1) expressing p210Bcr/Ab1 (lanes B, D, and E). Immunoprecipitations were performed with anti-ABL antibodies (lanes A and B), anti-rasGAP (lanes C and D) antibodies, or an anti-polyoma T antibody as a “nonimmune” antisera (lane E). The migration of protein molecular weight markers is indicated on the left. The migration of p120 rasGAP, p210Bcr/Ab1, and p140 c-ABL is indicated.
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this regard, it is noteworthy that expression of the IL-3 gene in marrow cells produces a myeloproliferative syndrome in mice with many of the features of CML. Expression of high levels of p210bcr/abl in murine myeloid cells is associated with conversion to factor independence, and it is possible that the phosphorylation of rasGAP contributes to loss of IL-3 dependence. However, it is of interest that other proteins we have previously associated with IL-3 signal transduction were observed to be constitutively phosphorylated on tyrosine in p210bcr/abl (+) cells.

rasGAP is found predominantly as a monomer in the cytosol of normal cells. However, in cells expressing an activated tyrosine kinase, such as pp60v-src, or cells stimulated with epidermal growth factor, rasGAP becomes phosphorylated on tyrosine and serine and forms a complex with two phosphoproteins, p62 and p190. The p62 and p190 are not recognized by rasGAP antisera, thus are only precipitated as a consequence of a physical interaction with rasGAP. In cell lines such as BV-173 and NALM-1, where tyrosine phosphorylation of rasGAP could be detected, rasGAP antiserum precipitated tyrosine phosphorylated p62 and p190, indicating complex formation between rasGAP and p62 and p190. In fact, tyrosine phosphorylation of partially purified rasGAP has no obvious effect on its activity; however, the rasGAP-p190 complex has been shown to have reduced rasGAP activity. Therefore, it is possible that tyrosine phosphorylation of rasGAP-associated proteins rather than tyrosine phosphorylation of rasGAP itself is responsible for activation of the ras pathway. The detergent used in these studies to extract proteins (1% NP40) does not efficiently extract proteins tightly associated with the cytoskeleton, and the presence, if any, of rasGAP-p210bcr/abl complexes associated with the cytoskeleton will need to be assessed by other techniques such as direct purification of cytoskeletal proteins.

The role of BCR-encoded sequences in transformation is unknown. Although BCR first-exon sequences specifically activate the p210bcr/abl tyrosine kinase, it is intriguing to consider the possibility that BCR could alter the substrate specificity of the ABL kinase or cause ABL to associate with new proteins. BCR sequences essential for transformation have recently been shown by Pendergast et al to bind to the ABL SH2 domain through an interaction that does not involve phosphorysine, and it will be of interest to investigate the role of BCR in the interaction of rasGAP and p210bcr/abl. The C-terminal region of BCR has recently been shown to have GTPase-activating activity for the ras-related GTP-binding protein, p21v ras, but not for p21v ras. Although the C-terminal region of BCR is not translocated to c-ABL in the Ph1 chromosome, it is possible that the N-terminal region of BCR, which is translocated, contains binding sites for proteins involved in p21v ras-mediated signal transduction. Also, since Campbell and Arlinghaus have recently demonstrated that p210bcr/abl forms a stable complex with p160 BCR, it is possible that p210bcr/abl could function, in part, through the p21v ras pathway. Thus, it is possible that the functions of rasGAP in signal transduction are altered not only by tyrosine phosphorylation of ABL, but also as a result of being in a complex with BCR or BCR-associated proteins.

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REFERENCES


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