Inhibition of the Bcr-Abl Oncoprotein by Bcr Requires Phosphoserine 354

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Abstract

The Bcr-Abl oncprotein is involved in the inhibition of oncogenic activity of the Bcr-Abl oncprotein. This inhibition is believed to be the result of binding to the SH2 domain of Bcr-Abl in a non-phosphotyrosine-dependent manner. We showed that the Arg to Leu mutation in the Phe-Leu-Val-Arg-Glu-Ser (FLVRES) sequence of the SH2 domain, known to interfere with phosphotyrosine sequence binding, did not block the binding of Bcr first exon sequences to the Abl SH2 domain. We examined the structural-functional properties of a first exon mutant of BCR lacking the oligomerization domain, termed Bcr(64–413), that encodes the Ser-Thr protein kinase activity of Bcr. The autokinase product contained a M, 45,000–47,000 and 55,000 protein. Both species were detected by a Bcr phosphoserine 354 sequence-specific antibody. In contrast, the S354A mutant of Bcr(64–413), although maintaining autokinase activity, produced only the M, 45,000–47,000 kinase product. Abl SH2 binding experiments indicated that the mouse, 55,000 species of Bcr(64–413) but not the 45,000–47,000 species bound strongly to glutathione transferase-Abl SH2. The S354A mutant of Bcr(64–413) did not bind to glutathione transferase-Abl SH2. An adenovirus encoding Bcr(64–413) S354A did not induce cell death in CML cell lines in contrast to wild-type Bcr(64–413). Our findings indicate that Ser-354 of Bcr is part of a gating mechanism, which, after its phosphorylation, allows structural changes to occur in the Bcr protein. This altered phosphoserine form of the Bcr protein selectively binds to the Abl SH2 domain of the oncprotein, which we propose down-regulates the activity of the Bcr-Abl tyrosine kinase.

Introduction

The Bcr-Abl oncprotein is generated from an abnormal chromosome 22 formed by the exchange of the ends of chromosome 9 and 22 (1). It results in the fusion of 5′ segments of the BCR gene to most of the ABL gene beginning at ABL exon 2. The role of Bcr sequences is now known to be important for the biological and biochemical activity of the Bcr-Abl oncprotein. The first 63 amino acids contain a coiled-coiled structure responsible for forming tetrameric forms of the Bcr-Abl oncprotein. This oligomerization event is the principal reason for the activation of the Abl kinase domain within the Bcr-Abl oncprotein (2).

A second important role for the cis-Bcr sequence concerns its ability to be phosphorylated by the Abl kinase domain (3). One of the residues phosphorylated is Tyr-177. This residue lies within a Grb2 SH2 binding consensus sequence [Tyr-Val-Asn-Val (YVNV)], which, when phosphorylated, stimulates the binding of the Grb2 adaptor protein and subsequent activation of the Ras pathway (4, 5). Ras activation by Bcr-Abl is required for myeloid leukemia induction in a mouse transplant model (6).

Three additional Bcr tyrosine residues are also phosphorylated as a result of the autophosphorylation of Bcr-Abl. Two of these Tyr residues are concerned with regulation of the Ser/Thr kinase activity of Bcr (7, 8). Phosphorylation of these two residues (Tyr-328 and Tyr-360), and possibly others, coincides with the down-regulation of Bcr Ser kinase activity (7).

We have shown that the Bcr protein antagonizes the oncogenic effects of the Bcr-Abl oncprotein (9). The inhibitory activity is localized within a Bcr deletion mutant [Bcr(64–413)] encoded by the first exon of BCR gene but lacking the oligomerization domain (9, 10). Experiments with either full-length Bcr or Bcr(64–413) indicate that the Ser-phosphorylated form of Bcr is the inhibitory form (8, 11). Importantly, induction of BCR expression at levels higher than the endogenous Bcr level blocks the oncogenic effects of Bcr-Abl in 80% of NOD/scid mice injected with a clone of K562 cells containing a tetracycline-off promoter to silence the exogenous BCR gene. In contrast, 100% of the mice died within 35 days of injection when BCR expression was not induced (11).

Materials and Methods

Cell Culture, Transient-transfection, and Bcr Kinase Assays. COS1 and K562 cells/BV173 cells were cultured in DMEM and RPMI medium, respectively, supplemented with 10% FCS (9). COS1 cells were transfected as described previously (9). Bcr Ser/Thr kinase assays were performed as described previously (9).

Adenovirus Infection. Recombinant replication defective adenovirus 5 encoding BCR(64–413), BCR(64–413) S354A, and the β Gal gene were prepared as described previously (10). K562 and BV173 cells were infected by a cell concentration method described by Wang et al. (10). Methods for β Gal staining were performed as described previously (10). K562 or BV173 cells were infected with a multiplicity of infection (MOI) of 12 for adenovirus BCR(64–413), 37 for adenovirus BCR(64–413) S354A, and 30 for adenovirus β Gal.

Antibodies and Western Blotting. Antipeptide antibodies were made in rabbits against Bcr peptides 1–16, 181–194, and 298–310. Antiphosphoserine 354 antibody was prepared in rabbits against a phosphoserine 354 Bcr peptide (QGSSRVpSPSTTTY) as described previously (12). Anti-Abl 8E9 is a mouse monoclonal antibody made against the SH2 domain of mouse c-Abl (13). Western blotting was performed as described previously (9).

Preparation and Expression of BCR(1–413). The wild-type and Arg to Leu (R to L) mutant SH2 sequences were subcloned into a pcDNA 3 vector containing a tag at the 3′ end. The R to L mutant of the SH2 domain was derived from a mutant SH2 domain of BCR-ABL (R to L mutant) provided by Bruno Calabretta (Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA). COS1 cells were cotransfected with either BCM1–413 and human ABL-SH2-RYIRS tag DNAs (14) or the epitope-tagged Abl SH2 containing the R to L mutant within the FLVRES5 sequence of the SH2 domain. Cells were lysed in Guo buffer (7) and the clarified extract mixed with the polyclonal tag antibody supplied by Dr. Sue-Hwa Lin of M. D. Anderson Cancer Center, Houston, TX (14). In contrast to the wild-type SH2 protein sequence, the mutated SH2 protein was not detected by the SH2 antibody, 8E9, which is a conformational antibody that detects the wild-type SH2 sequence (15). The immunoprecipitates were harvested on agarose A/G beads and the washed beads were eluted with SDS sample buffer and Western blotted with anti-Bcr (181–194).

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3 The abbreviations used are: FLVRES, Phe-Leu-Val-Arg-Glu-Ser; GSH, glutathione; GST, glutathione transferase; β Gal, β galactosidase.
Bcr sequences were not involved in binding to the Abl SH2 domain wild-type or the mutant SH2 protein, indicating that phosphotyrosine sequences were involved (termed A and B boxes), the implication is that phosphoserine sequences, not phosphotyrosine sequences, are being bound to the SH2 domain. Phosphosine-binding is consistent with the Bcr protein being a Ser/Thr protein kinase (16, 17). It is well known that an R to L mutation in the conserved FLVRES sequence of the SH2 domain blocks binding of phosphotyrosine sequences (18). We compared the binding of Bcr(1–413) or Bcr(64–413) S354A were lysed in Guo buffer (5 × 10^6 cells/ml) as above, incubated on ice for 30 min, and centrifuged at 100,000 × g for 40 min at 4°C. The supernatant fluid was incubated with 50–100 µg of either GST or GST-Abl SH2 produced in bacteria as described previously (15). GST/protein complexes were harvested by incubation for 4 h on ice, washed with kinase wash buffer (7) by suspension, and pelleting three times. Proteins were released from the beads by boiling in SDS sample buffer and removing beads by centrifugation in a table-top clinical centrifuge. Samples were processed by Western blotting as above.

**Results**

**BCR First Exon Sequences Bind to the R to L FLVRES SH2 Mutant of Abl.** Pendergast et al. (15) reported in 1991 that first exon sequences of Bcr bind to the Abl SH2 domain but surprisingly in a non-phosphotyrosine-dependent manner. Because Ser-rich Bcr sequences were involved (termed A and B boxes), the implication is that phosphoserine sequences, not phosphotyrosine sequences, are being bound to the Abl SH2 domain. Phosphosine-binding is consistent with the Bcr protein being a Ser/Thr protein kinase (16, 17). It is well known that an R to L mutation in the conserved FLVRES sequence of the SH2 domain blocks binding of phosphotyrosine sequences (18).

We compared the binding of Bcr(1–413) sequences, which contains the fully functional Bcr Ser/Thr kinase activity. The basic assay was incubation (7) but using ant-Bcr(181–194) and the immune complexes harvested with protein A-Sepharose beads. Duplicate reactions of either Bcr(64–413) S354A or Bcr(64–413) were incubated in the kinase assay and analyzed by SDS-PAGE; two lanes of each are shown. B, the lysate was processed for Western blotting with anti-Bcr(298–310).

**Fig. 2.** Bcr(64–413) is active as a Ser kinase and contains two distinct protein forms. A, COS1 cells were transiently transfected with BCR(64–413) S354A or BCR(64–413). Lysates were prepared and analyzed in the standard kinase assay (7) but using anti-Bcr(181–194) and the immune complexes harvested with protein A-Sepharose beads. Duplicate reactions of either Bcr(64–413) S354A or Bcr(64–413) were incubated in the kinase assay and analyzed by SDS-PAGE; two lanes of each are shown. B, the lysate was processed for Western blotting with anti-Bcr(298–310).

**Bcr(64–413) Forms Two Molecular Species in the Autokinase Reaction.** We had previously reported that a mutant form of Bcr first exon sequences which lack the oligomerization domain lack the Bcr function and contain two distinct protein forms. One had a mobility in SDS-PAGE similar to that expected for Bcr(64–413) (M, ~45,000–47,000; Fig. 2A, duplicate samples). The second form had a mobility ~55,000. The slower mobility of the M, ~55,000 form is suggestive of a hyper-Ser phosphorylated form of the M, 45,000 Bcr protein, but additional studies are needed to prove this point. Lysates of COS1 cells were also examined by Western blotting with anti-Bcr(298–310), and both forms of Bcr(64–413) were detected (Fig. 2B). Similar bands were detected with anti-Bcr(181–194) (see Fig. 3).

Analyses of the kinase reaction product of the S354A mutant of Bcr(64–413) detected only the M, 45,000 form (Fig. 2A, left panel) and not the M, 55,000 kDa form. Similarly, Western blotting with anti-Bcr(298–310) of COS1 cells expressing the S354A mutant did not detect the presence of the M, 55,000 form but did detect the M, 45,000 form of Bcr(64–413) S354A mutant (Fig. 2B). These results suggest that Ser-354 of Bcr is a critical amino acid required to produce this structural altered form of Bcr(64–413).
Western blotting was performed with anti-Bcr, which we have shown to be weakly detected by antiphosphoserine antibody (12). C. A block reactivity of antibody with phosphoserine Bcr sequences. PSPTTY to S antibody premixed with excess phosphoserine peptide (GQSSRVp) at position of a background band. B. PSPTTY S

Lane 3 413) S354A mutant; phosphorylated Bcr sequences (12). In contrast, neither band was detected by Western blotting with BCR (64–413) which was also detected in COS1 cells expressing the Bcr(64–413) wild-type lysate (Fig. 3A, Lane 2). This blot was developed with anti-Bcr(181–194). Identical results were obtained when blots from a similar experiment were probed with the anti-Bcr phosphoserine 354 sequence-specific antibody, indicating that it is the phosphoserine form of the M, 55,000 form of Bcr(64–413) that binds to the Abl-SH2 domain (not shown).

The S354A mutant of Bcr(64–413) Lacks Cell Death-inducing Activity for Bcr-Ab1-Positive CML Cell Lines. We have shown that Bcr(64–413) expression by infection with a recombinant adenovirus encoding BCR(64–413) blocked cell growth and induced cell death in Bcr-Ab1-positive CML cell lines and primary cultures of blood cells from CML patients with active disease (10). To determine what effect the S354A mutant had on K562 cells, we prepared a replication-defective adenovirus encoding S354A BCR(64–413), and compared its ability to induce cell death with that of adenovirus BCR(64–413). In these experiments, we counted dead cells (trypan blue-positive) and viable cells and presented the results as percentage of either viable cells or dead cells (Fig. 5). Infection of K562 cells showed that, whereas Bcr(64–413) induced cell death at 2, 5, and 8 days post-infection, the S354A mutant Bcr(64–413) had little effect on K562 cells (Fig. 5). Neither mock infection nor infection with β Gal-encoding adenovirus induced cell death in the K562 culture at significant levels (Fig. 5).

Under these same infection conditions, 70–80% of the cells stained positive for β Gal activity after infection with adenovirus encoding the β GAL gene (10). Expression of the Bcr(64–413) and the S354A mutant proteins were judged to be quite similar as determined by Western blotting with an anti-Bcr(181–194) antibody (Fig. 5, inset). Experiments performed with Bcr-Ab1-positive BV173 cells gave similar results in that cell death was not induced by expression of Bcr(64–413) S354A after adenovirus infection but was induced by Bcr(64–413) expression (not shown).

We note that efficient extraction of the M, 55,000 form of Bcr(64–413) requires mild detergent extraction (the absence of SDS detergent) prior to treatment of cells with SDS-containing sample buffer. Also, Bcr(64–413) is quite labile in the context of dying Bcr-Ab1-containing cells such as K562 cells; and, therefore, the Bcr(64–413) is completely degraded during the mild detergent extraction. Direct SDS sample buffer treatment of cells is required to extract Bcr(64–413) from K562 cells. Thus, the Fig. 5 inset shows only the M, 45,000–47,000 form of Bcr(64–413) and the S354A mutant.
The phosphotyrosine content of P210 BCR-ABL at day 2 after infection was reduced 39% in experiment 1 and 18% in Experiment 2, for an average reduction of 28% in adenovirus BCR(64–413)-infected cells compared with adenovirus BCR(64–413) S345A-infected K562 cells (not shown). Because equal number of viable cells were analyzed in the phosphotyrosine blots and because reduction of Bcr-Abl kinase activity by various treatments (e.g., the Abl kinase inhibitor STI-571) coincides with an increase in apoptosis and degradation of the Bcr-Abl protein (10, 19–21), we believe that this 28% reduction in Bcr-Abl phosphotyrosine content caused by Bcr(64–413) expression is an underestimate. The reduction of the phosphotyrosine content of P210 BCR-ABL in COS1 cells, which are not induced to undergo cell death after adenovirus/BCR(64–413) infection, is 35–40% (10).

Discussion

Our findings have confirmed the findings of Pendergast et al. (15), who first showed that non-phosphotyrosine Bcr sequences encoded by the first exon of the BCR gene bind tightly to the SH2 domain of Abl. Two Ser-rich Bcr sequences (termed A and B boxes) were involved in the binding to the SH2 domain of Abl. The implication from that study is that phosphoserine Bcr sequences bind firmly to the SH2 domain of Abl, a novel concept considering the well-known binding of phosphotyrosine-containing peptide sequences to SH2 domains (18). In their studies (15), deletion of the Ser-rich A and B boxes reduced the oncogenic activity of the BCR-ABL oncogene. This reduction was interpreted to mean that the Ser-rich boxes were required for efficient oncogenic activity. However, we believe that this interpretation is unlikely because these large deletions removed important amino acids such as Tyr-177, which is well known to be required for oncogenic activity (4, 5).

We confirmed that the binding of Bcr to the SH2 domain of Abl does not involve phosphotyrosine (Fig. 1). In these experiments, we observed that Bcr(1–413) bound tightly to both the wild-type Abl-SH2 and the R to L mutant of the SH2 domain of Abl (Fig. 1), a mutation known to interfere with the binding of phosphotyrosine sequences (18).

The phosphotyrosine-independent binding of proteins to SH2 domains has been reported in two other systems (22, 23) besides the Bcr/Abl SH2 system (15). One is the interaction of Raf-1 with the SH2 domains of Fyn and Src (22); the other is the interaction of a Mu Mu

![Image](https://example.com/image1.png)
(Fig. 3). On the basis of its retarded mobility in denaturing SDS gels compared with the expected size of Bcr(64–413), the M₆₅,500 form of Bcr(64–413) appears to have structural changes that could be the result of hyperphosphorylation on Ser residues, possibly those within the Ser-rich A and B boxes (15). It is known that the hyperphosphorylation of proteins on Ser residues causes retarded mobility in SDS polyacrylamide gels (24).

Our findings show that Ser-354 is required for the formation of the M₆₅,500 Bcr species (Figs. 2 and 3). We also found, both in kinase/peptide mapping assays performed on Bcr proteins (not shown) and by detection of the intracellular Bcr protein by the phosphospecific 354 Bcr sequence-specific antibody (Fig. 3; Ref. 12), that Ser-354 of Bcr is a major site of Bcr phosphorylation. These findings establish that Ser-354 of Bcr is an authentic phosphorylation site. Similarly, Ser 356 was also found to be a phosphorylation site (12). Furthermore, preliminary studies with Bcr peptides surrounding these two residues suggest that phosphorylation of Ser-354 is required for subsequent phosphorylation of Ser 356.

The functional importance of the M₆₅,500 phosphosine form of the Bcr protein is evident from its ability to bind to the SH2 of Ab, whereas the M₆₅,45,000 form of Bcr(64–413) did not bind (Fig. 4). Our previous findings (9) that Bcr(64–413) is resistant to Tyr phosphorylation by c-AbI and Bcr-AbI is consistent with the lack of phosphotyrosine involvement in this Ab SH2 binding. We propose that phosphospecific Bcr binding would likely perturb the SH2 domain and, therefore, would inhibit the Tyr kinase activity (9–11) and the oncogenic effects (growth and survival) of the Bcr-Ab oncprotein, which is what we have observed in studies with adenoviruses-encoding Bcr(64–413) (Fig. 5; Ref. 10). However, when binding to the SH2 domain is prevented, as is the case with the S354A mutant that fails to form the M₆₅,500 form, no inhibition of the oncogenic activity of Bcr-AbI was observed (Fig. 5).

Our earlier studies indicated that phosphosine 354 is required for the Bcr-AbI kinase inhibitory effects observed with a short Bcr peptide (350-SSRvPSPTTYRMFRDK-366; Ref. 9). This phosphopeptide, but not the unphosphorylated form, effectively inhibits the Bcr-AbI and AbI kinases in vitro in a dose-dependent manner. Similarly, immune complexes that contain Bcr(64–413) also strongly inhibit the Bcr-AbI kinase (9).

Another issue is whether the full-length BCR gene product, P160 BCR, also has an altered form equivalent to the M₆₅,500 form of Bcr(64–413). Our experience has been that P160 BCR is detected as a closely spaced doublet in denaturing SDS polyacrylamide gels (25). In other studies, we sometimes detected, by Western blotting with anti-Bcr(298–310) of COS1 cells transfected with full-length BCR, a more slowly migrating form of Bcr (M₆₅,180,000) in addition to the M₆₅,160,000 form (12). Also, in Bcr kinase assays, we have routinely detected a M₆₅,180,000 size form of the M₆₅,160,000 Bcr protein. Of interest, Western blotting of blood cells from CML patients with the phosphosine 354 sequence-specific Bcr antibody detected a clear signal of the M₆₅,160,000 Bcr protein, which was specifically blocked by excess phosphosine 354 Bcr peptide (26).

The findings presented here indicate that an altered phosphosine Bcr structure is involved in the binding to the SH2 domain of AbI. We are currently investigating the structure of the M₆₅,500 phosphosine form of Bcr to the SH2 domain of Ab, and how the binding of phosphosine Bcr to the SH2 domain antagonizes the oncogenic effects of the Bcr-Ab oncprotein.

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4 J. Liu and R. B. Arlinghaus, unpublished observations.
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