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Advances in Brief

Inhibition of the Bcr-Abl Oncoprotein by Bcr Requires Phosphoserine 354[1]

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Abstract

The BCR protein is involved in the inhibition of oncogenic activity of the Bcr-Abl oncoprotein. 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_r 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_r 45,000–47,000 kinase product. Abl SH2 binding experiments indicated that the M_r 55,000 species of Bcr(64–413) but not the M_r 45,000–47,000 species bound strongly to glutathime transferase-Abl SH2. The S354A mutant of Bcr(64–413) did not bind to glutathime 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 oncoprotein, which we propose down-regulates the activity of the Bcr-Abl tyrosine kinase.

Introduction

The Bcr-Abl oncoprotein 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 oncoprotein. The first 63 amino acids contain a coiled-coiled structure responsible for forming tetrameric forms of the Bcr-Abl oncoprotein. This oligomerization event is the principal reason for the activation of the Abl kinase domain within the Bcr-Abl oncoprotein (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 oncoprotein (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(7).

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 (GQSSRVpSPSPTTY) 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 BCR(1–413) and human ABL-SH2-RYIRS tag DNAs (14) or the epitope-tagged Abl SH2 containing the R to L mutant within the FLVRES[3] 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 (13). 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, glutathime transferase; β Gal, β galactosidase.

Bcr(64–413) Studies. Lysates from COS1 cells (10×10^6 cells/ml of Guo lysis buffer (7) expressing Bcr(64–413) were incubated for 30 min on ice in the Guo Buffer lysis solution containing a cocktail of protease and phosphatase inhibitors (7) including the protease inhibitor, 4(2-aminoethyl)benzene-sulfonyl fluoride (AEBSF; Sigma Chemical Co., St. Louis, MO) prior to processing for Western blotting. The sample was centrifuged at $100,000 \times g$ for 40 min at 4°C either prior to immunoprecipitation with anti-Bcr or prior to adding equal volumes of 2× SDS sample buffer and boiling.

GST-Abl SH2 Pull-Down Assays. COS1 cells expressing either Bcr(64–413) 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 $100,000 \times g$ 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. Phosphoserine-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, either to wild-type or to the R to L FLVRES mutant of Abl SH2 sequences (Fig. 1). Lysates of transfected COS1 cells expressed similar levels of either wild-type or R to L Abl SH2, as judged by Western blotting of the approximately M_r 10,000 epitope-tagged SH2 protein (not shown). The SH2 protein sequences were immunoprecipitated from the COS1 lysate with antibody to the tagged epitope attached to the Abl-SH2 sequences, and the washed immunoprecipitates were eluted with hot SDS sample buffer and Western blotted with anti-Bcr(181–194). No difference was observed in the binding of Bcr(1–413) to either the wild-type or the mutant SH2 protein, indicating that phosphotyrosine Bcr sequences were not involved in binding to the Abl SH2 domain under these conditions. Moreover, these data indicate that the phosphotyrosine binding motif of the SH2 domain is not involved in binding phosphoserine-containing Bcr to the SH2 domain.

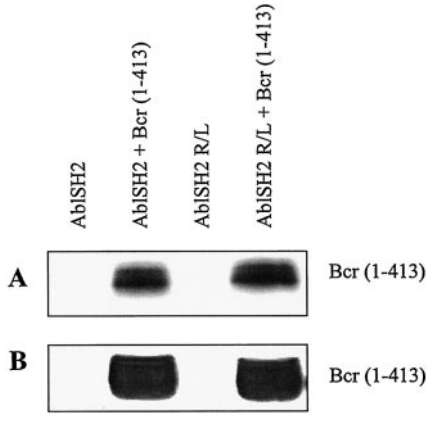

Fig. 1. Bcr(1–413) binds to the FLVRES mutant (R to L) of the SH2 domain of Abl. *A*, COS1 cells were transfected with either wild-type ABL-SH2-epitope-tagged DNA or the ABL-SH2-epitope-tagged FLVRES mutant (R to L) alone or were cotransfected with BCR(1–413) DNA. Lysates were immunoprecipitated with the tag antibody and were Western blotted with anti-Bcr(181–194). *B*, the total lysate was Western blotted with anti-Bcr(181–194).

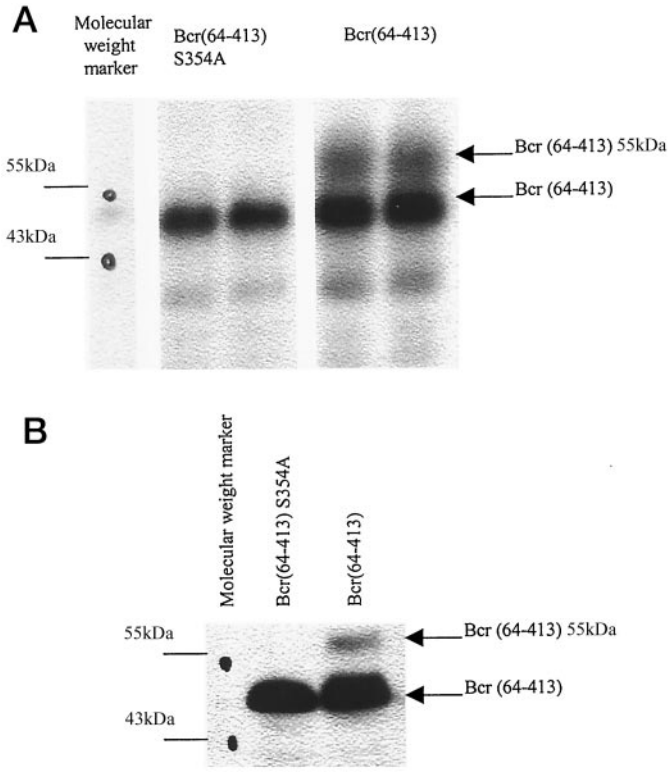

Fig. 2. Bcr(64–413) is active as a Ser kinase and contains two distinct protein forms. *A*, COS1 cells were transiently transfected with BCR(64–413) S354A or BCR(64–413). Lysates were prepared and analyzed in the standard kinase assay (7) but using anti-Bcr(181–194) and the immune complexes harvested 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 that lack the oligomerization domain is resistant to Tyr phosphorylation by activated c-Abl. Furthermore, Bcr(64–413) inhibits the oncogenic activity of Bcr-Abl (9, 10). Therefore, we wanted to determine whether the Ser protein kinase activity was still functional in Bcr(64–413) and whether there were any interesting properties associated with this mutant protein. We found that Bcr(64–413) maintained significant amounts of kinase activity despite lacking the oligomerization domain. SDS gel electrophoresis of the autokinase product surprisingly revealed two molecular forms. One had a mobility in SDS-PAGE similar to that expected for Bcr(64–413) (M_r ~45,000–47,000; Fig. 2*A*, duplicate samples). The second form had a mobility of M_r ~55,000. The slower mobility of the M_r 55,000 form is suggestive of a hyper-Ser phosphorylated form of the M_r 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. 2*B*). 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_r 45,000 form (Fig. 2*A*, *left panel*) and not the M_r 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 a M_r 55,000 form but did detect the M_r 45,000 form of Bcr(64–413) S354A mutant (Fig. 2*B*). These results suggest that Ser-354 of Bcr is a critical amino acid required to produce this structural altered form of the Bcr(64–413).

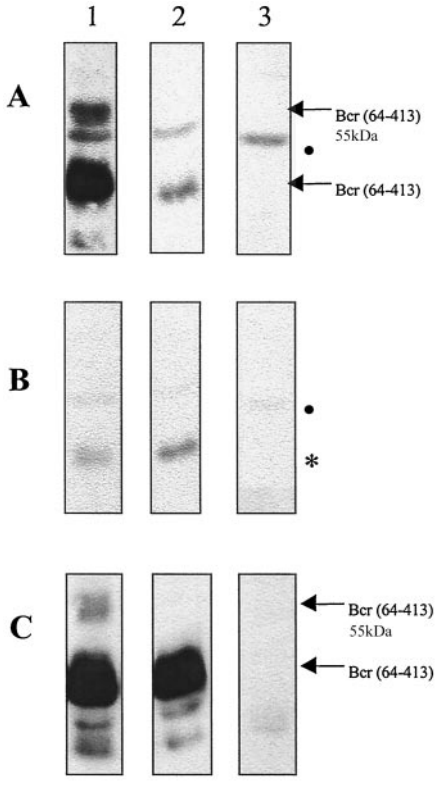

Fig. 3. Detection of the M_r 55,000 form of Bcr(64–413) by Western blotting with Bcr antiphosphoserine 354 sequence specific antibody. COS1 cells were transiently transfected with either BCR(64–413) or BCR(64–413) S354A. Cell were lysed in a detergent-containing medium (see "Materials and Methods") before analysis by Western blotting. *Lane 1*, COS1 cells expressing Bcr(64–413); *Lane 2*, COS1 cells expressing the Bcr(64–413) S354A mutant; *Lane 3*, vector-transfected COS1 cells. *A*, Western blotting with anti-Bcr phosphoserine 354 antibody mixed with excess Bcr peptide GQSSRV**S**PSPTTY to block reactivity with the antibodies to nonphosphorylated Bcr sequences. ●, the position of a background band. *B*, Western blotting with anti-Bcr phosphoserine 354 antibody premixed with excess phosphoserine 354 peptide (GQSSRVp**S**PSPTTY) to block reactivity of antibody with phosphoserine 354 Bcr sequences. ●, a background band as in *A*; ∗, a cross-reactive protein that we believe to be phosphoserine 356 Bcr(64–413), which we have shown to be weakly detected by antiphosphoserine 354 antibody (12). *C*, Western blotting was performed with anti-Bcr(298–310).

Sequence-specific Phosphoserine Antibody Detects Bcr(64–413). We have developed an antipeptide antibody that specifically detects the phosphoserine 354 Bcr sequence (12). The M_r 45,000 and 55,000 forms of Bcr(64–413) were detected with Bcr phosphoserine 354 sequence-specific antibody (Fig. 3*A*, *Lane 1*). These immunoblots were performed with excess amounts of the nonphosphorylated Bcr peptide to block any contribution by antibodies directed at nonphosphorylated Bcr sequences (12). In contrast, neither band was detected by the antibody premixed with excess phosphoserine 354 peptide (Fig. 3*B*, *Lane 1*), which indicated that the antibody was detecting only the phosphoserine 354-containing sequence in both the M_r 45,000 and the 55,000 forms. As expected, the S354A mutant of Bcr(64–413) was not detected by the Bcr phosphoserine 354 sequence-specific antibody (Fig. 3*A*, *Lane 2*). Only a minor background band was present in the Bcr(64–413) S354A lysate, which migrated between the M_r 45,000 and 55,000 forms of Bcr(64–413); which was also detected in COS1 cell lysate (Fig. 3*A*, *Lane 3*, ●) and the Bcr(64–413) wild-type lysate (Fig. 3, *Lanes 1*). Of interest, a second minor band that migrates slightly faster than the M_r 45,000 form of Bcr(64–413) was detected in both Bcr(64–413) and Bcr(64–413) S354A lysates (Fig. 3*A*, *Lane 2*, and Fig. 3*B*, *Lanes 1* and *2*, ∗). Although the identity of this protein is unknown, it does not appear to be a background band (it is absent in COS1 cells, Fig. 3*B*, *Lane 3*). It might be the phosphoserine 356 form of Bcr(64–413) and the S354A mutant, because we have shown that the antiphosphoserine 354 Bcr antibody cross-reacts with the phosphoserine 356 sequence (the sequence in this region is 350-SSRV**SPSP**TTY-360; Ref. 12). The loading control for this experiment is shown in Fig. 3*C* in which the anti-Bcr(181–194) antibody strongly detected the M_r 45,000 and 55,000 forms of Bcr(64–413).

The M_r 55,000 Form of Bcr(64–413) Binds to the Abl-SH2 Domain. Because Bcr first exon sequences are known to bind to the Abl SH2 domain in a non-phosphotyrosine-dependent manner (15), we performed Abl SH2 binding experiments with Bcr(64–413). For these experiments, COS1 cells were transiently transfected with either BCR(64–413) S354A or BCR(64–413). Cells were lysed and mixed with various concentrations of either GST-Abl SH2 beads or GST beads. After incubation, the GST-Abl SH2 complexes were harvested by pull down assays with GSH-agarose beads and the bound proteins were analyzed by SDS gel electrophoresis. The optimal amount of GST-Abl SH2 beads was determined in separate experiments to be 50–100 μg of beads. Using this amount we compared GST-Abl SH2 with GST only (Fig. 4). The results showed that only the M_r 55,000 form of Bcr(64–413) bound to the Abl SH2 domain (Fig. 4, *Lane 6*); no M_r 45,000 form of Bcr(64–413) was bound. The S354A mutant, which contained only the M_r 45,000 protein, did not bind to GST-Abl SH2 (Fig. 4, *Lane 4*). Neither Bcr(64–413) nor the S354A mutant of Bcr(64–413) bound to GST alone (Fig. 4, *Lanes 5* and *3*, respectively). The loading control for these binding experiments is shown in Fig. 4, *Lanes 1* and *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_r 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-Abl-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-Abl-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 postinfection, 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-Abl-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_r 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-Abl-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_r 45,000–47,000 form of Bcr(64–413) and the S354A mutant.

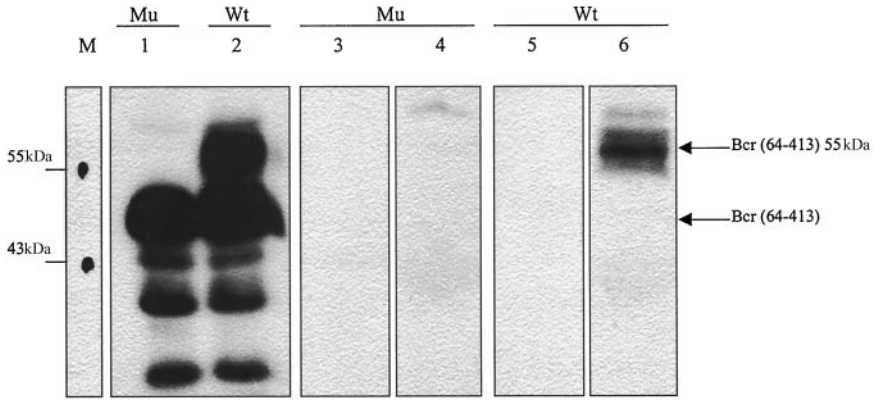

Fig. 4. The M_r 55,000 form of Bcr(64–413) binds to GST-Abl SH2. In these experiments COS1 cells were lysed in Guo buffer and incubated with GST or GST-Abl SH2 pull-down methods and Western blotted with anti-Bcr(298–310) as described in "Materials and Methods." *Lane 1*, the total lysate of COS1 cells expressing Bcr(64–413) S354A; *Lane 2*, the total lysate COS1 cells expressing Bcr(64–413); *Lane 3*, lysate of Bcr(64–413) S354A incubated on ice with 50 μg of GST beads (the bound portion was eluted with boiling SDS sample buffer); *Lane 4*, lysate of Bcr(64–413) S354A incubated with 50 μg of GST-Abl SH2 beads (the bound portion was eluted with boiling SDS sample buffer); *Lane 5*, lysate of Bcr(64–413) incubated with 50 μg of GST beads (the bound portion was eluted with boiling SDS sample buffer); *Lane 6*, lysate of Bcr(64–413) incubated with 50 μg of GST-Abl SH2 beads, eluted, and Western blotted with anti-Bcr(298–310). *Lane M*, molecular weight markers in thousands. *Across top of the Lanes*: *Mu*, mutant Bcr(64–413) S354A; *Wt*, wild-type Bcr(64–413).

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).

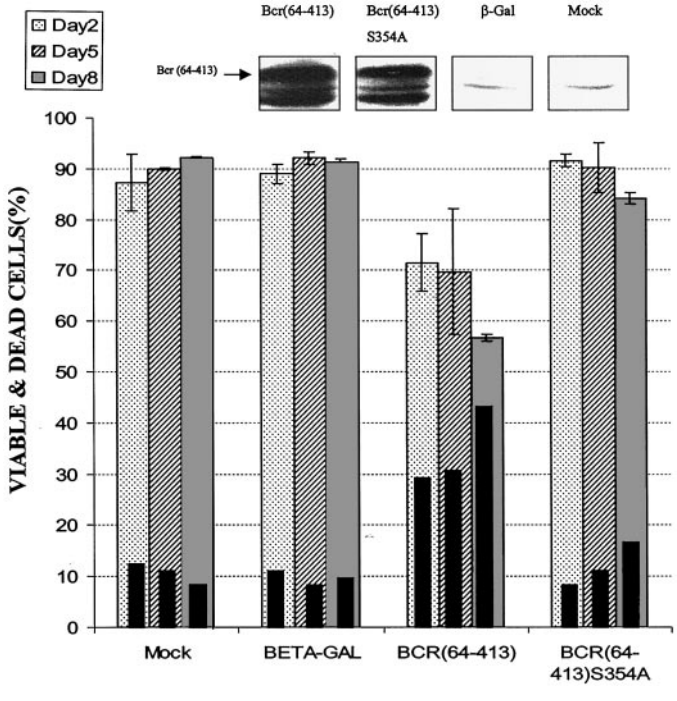

Fig. 5. BCR(64–413) S354A is deficient in its ability to induce cell death in K562 cells. Viable K562 cells (▨, ▨, ▨) and dead K562 cells (■) were measured by trypan blue staining. Cells counts were the average of triplicates. Each viability and dead-cell value is the average of two separate adenovirus infection experiments. *Inset*, Western blot analysis of adenovirus-infected K562 cells at 8 days postinfection with anti-Bcr(181–194). *Arrow*, the intact form of the M_r 45,000–47,000 Bcr(64–413) protein.

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 M_r 62,000 protein with the SH2 domain of p56 LCK (23). Of interest the phosphorylation of Ser 59 of p56 LCK, which is upstream of the SH2 domain of p56 LCK, is thought to regulate phosphotyrosine binding to the LCK SH2 domain (23).

Importantly, we found that a unique form of Bcr [the M_r 55,000 species of Bcr(64–413)] that lacks the oligomerization domain, was bound to the SH2 domain of Abl (Fig. 4). Bcr(64–413) is not expected to form tetramers because it lacks the coiled-coil domain that is required for this oligomerization (2). Of interest, the Bcr(64–413) mutant existed as two types of proteins with sizes of M_r 45,000 and 55,000, which were separable by denaturing SDS gel electrophoresis. (Fig. 2). Both forms of Bcr(64–413) contained phosphoserine 354

(Fig. 3). On the basis of its retarded mobility in denaturing SDS gels compared with the expected size of Bcr(64–413), the M_r 55,000 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 M_r 55,000 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 phosphoserine 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.[4]

The functional importance of the M_r 55,000 phosphoserine form of the Bcr protein is evident from its ability to bind to the SH2 of Abl whereas the M_r 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-Abl and Bcr-Abl is consistent with the lack of phosphotyrosine involvement in this Abl SH2 binding. We propose that phosphoserine 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-Abl oncoprotein, 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 M_r 55,000 form, no inhibition of the oncogenic activity of Bcr-Abl was observed (Fig. 5).

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

Another issue is whether the full-length BCR gene product, P160 BCR, also has an altered form equivalent to the M_r 55,000 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 (M_r ~180,000) in addition to the M_r 160,000 form (12). Also, in Bcr kinase assays, we have routinely detected a M_r 180,000 size form of the M_r 160,000 Bcr protein.[4] Of interest, Western blotting of blood cells from CML patients with the phosphoserine 354 sequence-specific Bcr antibody detected a clear signal of the M_r 160,000 Bcr protein, which was specifically blocked by excess phosphoserine 354 Bcr peptide (26).

The findings presented here indicate that an altered phosphoserine Bcr structure is involved in the binding to the SH2 domain of Abl. We are currently investigating the structure of the M_r 55,000 form of Bcr(64–413), the mechanism of this unique binding of the M_r 55,000 phosphoserine form of Bcr to the SH2 domain of Abl, and how the binding of phosphoserine Bcr to the SH2 domain antagonizes the oncogenic effects of the Bcr-Abl oncoprotein.

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

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