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An actin-binding function contributes to transformation by the Bcr-Abl oncoprotein of Philadelphia chromosome-positive human leukemias

John R.McWhirter and Jean Y.J.Wang[1]

Department of Biology and Center for Molecular Genetics, University of California at San Diego, La Jolla, CA 92093-0116, USA

[1]Corresponding author

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In Philadelphia chromosome-positive human leukemias, which include chronic myelogenous leukemia and some acute lymphocytic leukemias, the c-*abl* proto-oncogene on chromosome 9 becomes fused to the *bcr* gene on chromosome 22, and Bcr-Abl fusion proteins are produced. The Bcr sequences activate the Abl tyrosine kinase which is required for the transforming function of Bcr-Abl. The Bcr sequences also enhance an F-actin-binding activity associated with c-Abl. Here, we show that binding of c-Abl and Bcr-Abl proteins to actin filaments *in vivo* and *in vitro* is mediated by an evolutionarily conserved domain at the C-terminal end of c-Abl. The c-Abl F-actin-binding domain contains a consensus motif found in several other actin-crosslinking proteins. Mutations in the consensus motif are shown to abolish binding to F-actin. Bcr-Abl proteins unable to associate with F-actin have a reduced ability to transform Rat-1 fibroblasts and to abrogate the requirement for interleukin-3 in the lymphoblastoid cell line Ba/F3. In transformed cells, Bcr-Abl induces a redistribution of F-actin into punctate, juxtanuclear aggregates. The binding to actin filaments has important implications for the pathogenic and physiological functions of the Bcr-Abl and c-Abl proteins.

Key words: c-*abl*/chronic myelogenous leukemia/cytoskeleton/interleukin-3/protein-tyrosine kinase

Introduction

The product of the c-*abl* proto-oncogene is a non-receptor protein-tyrosine kinase that is ubiquitously expressed in mammalian cells. The c-Abl protein contains at least four functional domains: an N-terminal variable domain encoded by two alternative first exons (called Type I and Type IV in mouse c-*abl*, or Type 1a and 1b in human c-*abl*), a kinase regulatory domain which includes two regions of homology to the *src* tyrosine kinase (src homology regions 3 and 2, or SH3 and SH2), the tyrosine kinase domain, and a large C-terminal segment that is unique to the Abl subfamily of tyrosine kinases [Figure 2, c-Abl(IV)]. When the c-Abl protein is overproduced in fibroblasts, it is localized in both the nucleus and the cytoplasm (Van Etten *et al.*, 1989). The nuclear localization of the c-Abl protein is determined primarily by the C-terminal segment, which has been shown to contain a nuclear translocation signal and a DNA binding domain (Van Etten *et al.*, 1989; Kipreos and Wang, 1992).

The results of many studies indicate that oncogenic activation of tyrosine kinases requires the deregulation of the kinase activity, and the targeting of the kinase to specific subcellular locations (reviewed by Cantley *et al.*, 1991). Many oncogenic kinases contain an N-terminal sequence which can be modified by addition of the fatty acid myristate. These *N*-myristoylation signals are believed to target the kinases to cellular membranes and, in some cases, to specific membrane receptors (reviewed by Resh, 1990). In the Gag-v-Abl protein of Abelson murine leukemia virus, the Abl tyrosine kinase is deregulated by deletion of the variable and SH3 domains, and an *N*-myristoylation signal is provided by the retroviral Gag sequence (Franz *et al.*, 1989; Jackson and Baltimore, 1989). Because the Type IV c-Abl protein already contains an *N*-myristoylation signal, it can be converted into an oncogene simply by mutating the SH3 domain (Franz *et al.*, 1989; Jackson and Baltimore, 1989).

The c-*abl* gene is activated by an entirely different mechanism in Philadelphia chromosome (Ph^1)-positive human leukemias, in which a chromosome translocation causes the c-*abl* gene on chromosome 9 to become fused to the *bcr* gene on chromosome 22 (Heisterkamp *et al.*, 1983; Hermans *et al.*, 1987; Shtivelman *et al.*, 1985). This results in the synthesis of Bcr-Abl fusion proteins in which the variable domain of c-Abl is replaced by N-terminal Bcr sequences. In chronic myelogenous leukemia (CML), the fusion protein contains 902 or 927 amino acids of Bcr, whereas in Ph^1-positive acute lymphocytic leukemia (ALL), it contains only 426 amino acids of Bcr. The Bcr-Abl proteins differ in two important ways from the Gag-v-Abl protein: (i) their SH3 regions are intact and (ii) they are not *N*-myristoylated. The oncogenic activity of Bcr-Abl proteins is generally weaker than that of Gag-v-Abl. Unlike Gag-v-Abl, Bcr-Abl cannot transform NIH3T3 fibroblasts (Daley *et al.* 1987). Bcr-Abl can cooperate with the *myc* oncogene to transform Rat-1 fibroblasts but with much lower efficiency than Gag-v-Abl (Lugo and Witte, 1989; Lugo *et al.*, 1990). Although Bcr-Abl can stimulate the growth of primary bone marrow cells, the cells often fail to become fully transformed (McLaughlin *et al.*, 1989). It is likely that these differences in transforming activity between Gag-v-Abl and Bcr-Abl are due at least in part to differences in subcellular localization.

Previous results have shown that the Bcr sequences not only deregulate the Abl tyrosine kinase, but also activate an actin filament-binding function associated with c-Abl (McWhirter and Wang, 1991). Two regions of Bcr have been shown to be essential for the transforming function of Bcr-Abl (Figure 2, Bcr-Abl). Region 1 has been mapped to amino acids 1-63 and region 2 to amino acids 176-242 (McWhirter and Wang, 1991; Muller *et al.* 1991; J.R.McWhirter and J.Y.J.Wang, unpublished data). Region 2 may contribute to the deregulation of the tyrosine kinase by binding to the Abl SH2 domain (Pendergast *et al.*, 1991). Region 1 deregulates the tyrosine kinase and also enhances the actin-binding function of Abl (McWhirter and Wang, 1991). The enhancement of the actin-binding function by region 1 is independent of its effect on the tyrosine kinase

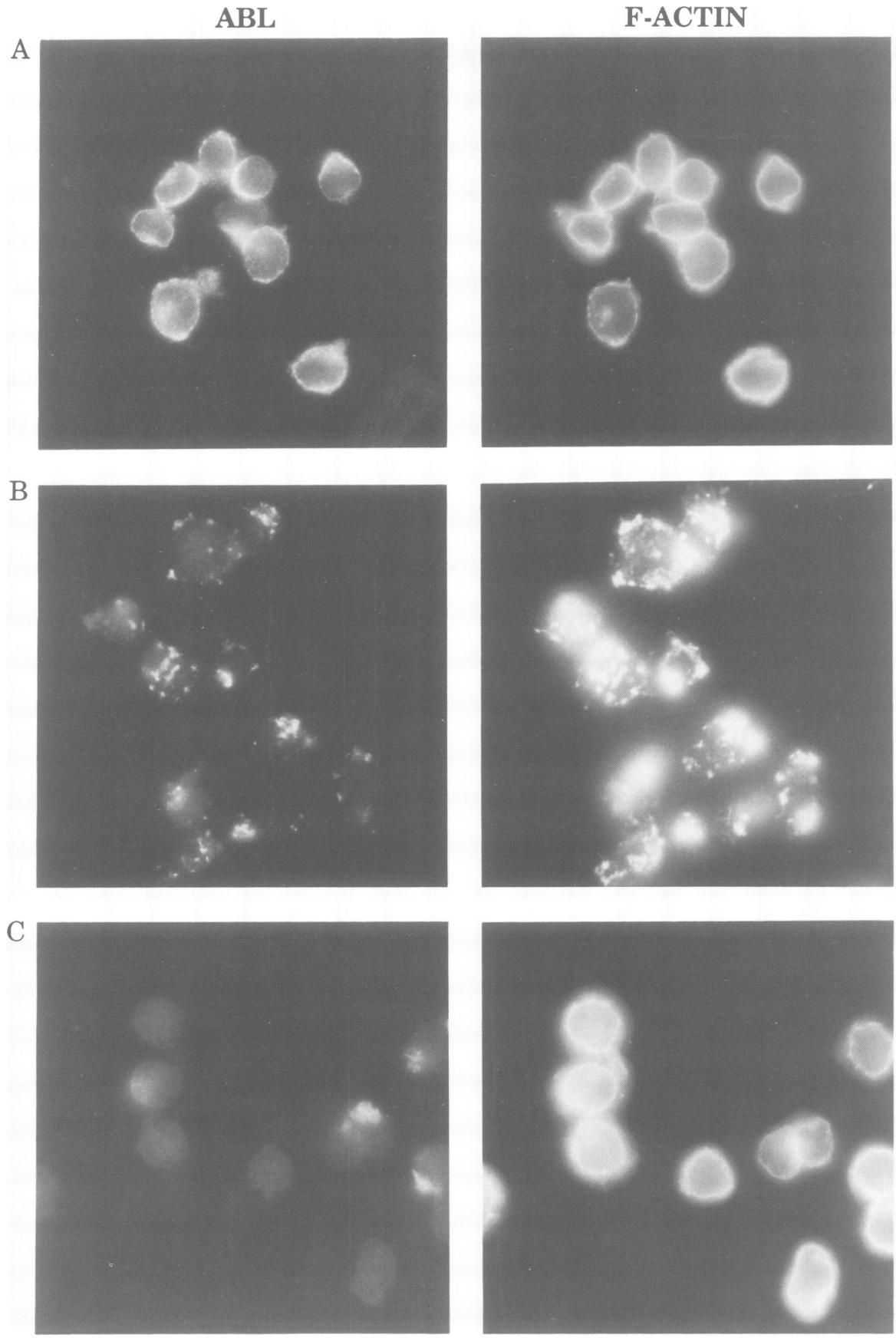


Fig. 1. Colocalization of p210$^{bcr\text{-}abl}$ with F-actin in K562 cells. K562 cells were prepared for immunofluorescence as described in Materials and methods. Primary antibody was anti-Abl 8E9. Secondary antibody was rhodamine-conjugated goat anti-mouse. FITC-conjugated phalloidin was added with the secondary antibody to visualize F-actin. (**A**) Untreated cells; (**B**) cells treated with 20 μg/ml cytochalasin B for 30 min before fixation; (**C**) untreated cells stained without the primary anti-Abl antibody. Left-hand panels: rhodamine signal showing ABL localization. Right-hand panels: fluorescein signal showing F-actin localization.

(McWhirter and Wang, 1991). The finding that a region of Bcr required for transformation is also required for the activation of the actin-binding function suggests that actin-binding may contribute to transformation.

To determine the role of actin-binding in transformation by Bcr-Abl, we have mapped the F-actin-binding domain and have shown that this domain contributes to the transforming activity of Bcr-Abl.

Results

p210$^{bcr-abl}$ is associated with actin filaments in a CML cell line

To confirm that Bcr-Abl is associated with actin filaments in leukemic cells, the localization of p210$^{bcr-abl}$ was examined by indirect immunofluorescence before or after treatment of K562 cells with cytochalasin B (Figure 1). Untreated and cytochalasin-treated K562 cells were double-stained with an antibody for Abl (to detect p210$^{bcr-abl}$) and with phalloidin (to detect F-actin). In untreated K562 cells, p210 colocalized with F-actin in the cortical cytoskeleton (Figure 1A). The general cytoplasmic staining was non-specific, as it was observed when anti-Abl was omitted from the staining procedure (Figure 1C). Identical results were obtained when the cells were extracted with Triton X-100 prior to fixation, so the cortical staining was not due to association of p210 with the plasma membrane (data not shown). In cells treated with cytochalasin B, both F-actin and p210 became reorganized into dots and patches, and remained colocalized (Figure 1B). Similar results were obtained for a kinase-defective Bcr-Abl expressed in COS cells. This protein contained the first 509 amino acids of Bcr fused to a mouse c-Abl protein containing a Lys → His point mutation in the ATP-binding site (McWhirter and Wang, 1991). It colocalized with both actin stress fibers and actin meshwork in untreated cells and remained colocalized with F-actin dots and patches after cytochalasin treatment (data not shown).

This result establishes that the subcellular localization of p210 in K562 cells is similar to that of our recombinant Bcr-Abl fusion protein in fibroblasts. Thus, Bcr-Abl can associate with F-actin in different cell types and the actin-binding function is conserved between the mouse and human c-Abl proteins.

Association of Bcr-Abl with actin filaments requires sequences at the C-terminal end of Abl

To identify regions required for association of Bcr-Abl with actin filaments, deletions were made in a Bcr-Abl fusion that contained the first 509 amino acids of Bcr and common amino acids 5−1097 of mouse c-Abl (Figure 2, Bcr-Abl). The effect of these deletions on association of the protein with actin in COS cells was determined by immunofluorescence. We have shown previously that deletion of amino acids 586−1097 of c-Abl from Bcr-Abl eliminates association with actin (Figure 2, Bcr-Abl/ΔNar; McWhirter and Wang, 1991). The association with F-actin was found to be eliminated with shorter deletions which removed amino acids 935−1097 (ΔSal), 1066−1097 (ΔBgl) or 1095−1097 (ΔTth) of c-Abl (Figure 2). Insertion of a *Pvu*I linker into the unique *Tth*111I restriction site, adding three amino acids (Arg-Ser-Val) between c-Abl amino acids 1094 and 1095,

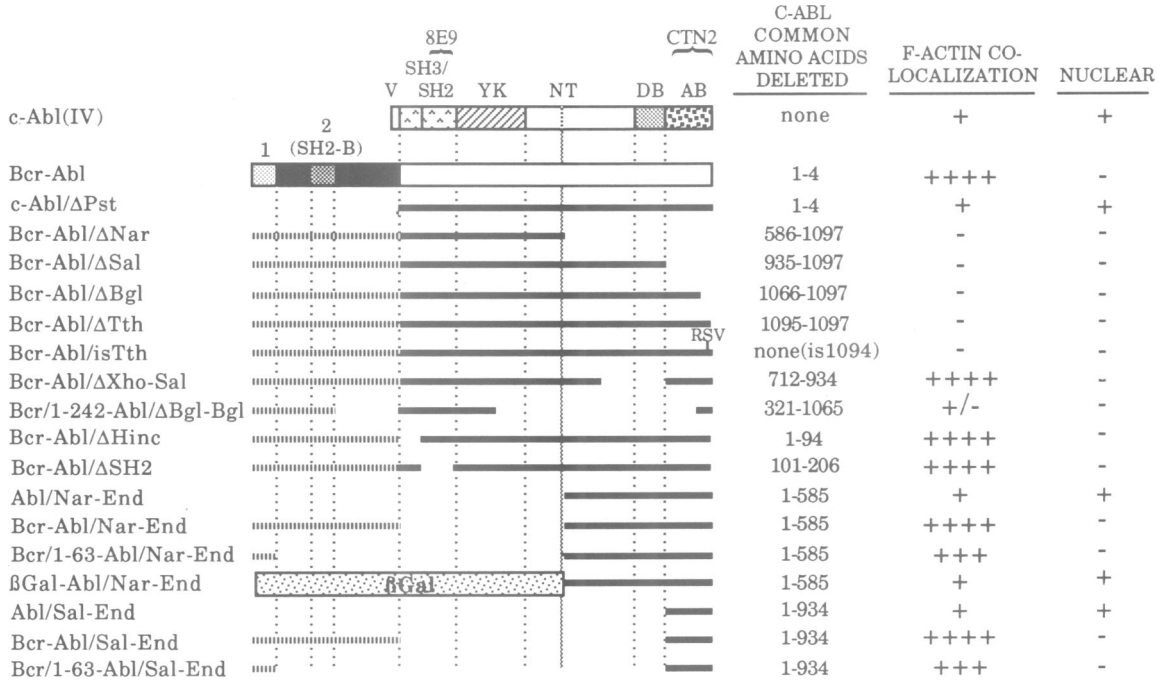


Fig. 2. Summary of constructs and subcellular localizations. c-Abl(IV): V, variable domain; SH3/SH2, src-homology domains 3 and 2; YK, tyrosine kinase domain; NT, nuclear translocation signal; DB, DNA-binding domain; AB, actin-binding domain; 8E9 and CTN2, regions recognized by the two anti-Abl antibodies used for immunostaining. Bcr-Abl: black box, Bcr amino acids 1−509; white box, Abl amino acids 5−1097; 1, Bcr region one; 2 (SH2-B), Bcr region two (SH2-binding domain). Deletion, insertion and fusion mutants were made as described in Materials and methods. Dashed lines, Bcr sequences; black lines, Abl sequences; stippled box, β-galactosidase sequences. F-actin colocalization: ++++, strong colocalization with actin bundles plus little or no diffuse staining; +++, strong colocalization with actin bundles plus some diffuse staining; +, weak colocalization with actin bundles plus strong diffuse staining; +/−, colocalization with ends of actin bundles plus strong diffuse staining; −, no detectable colocalization with actin bundles. Nuclear: +, strong nuclear staining in most cells; −, little or no nuclear staining.

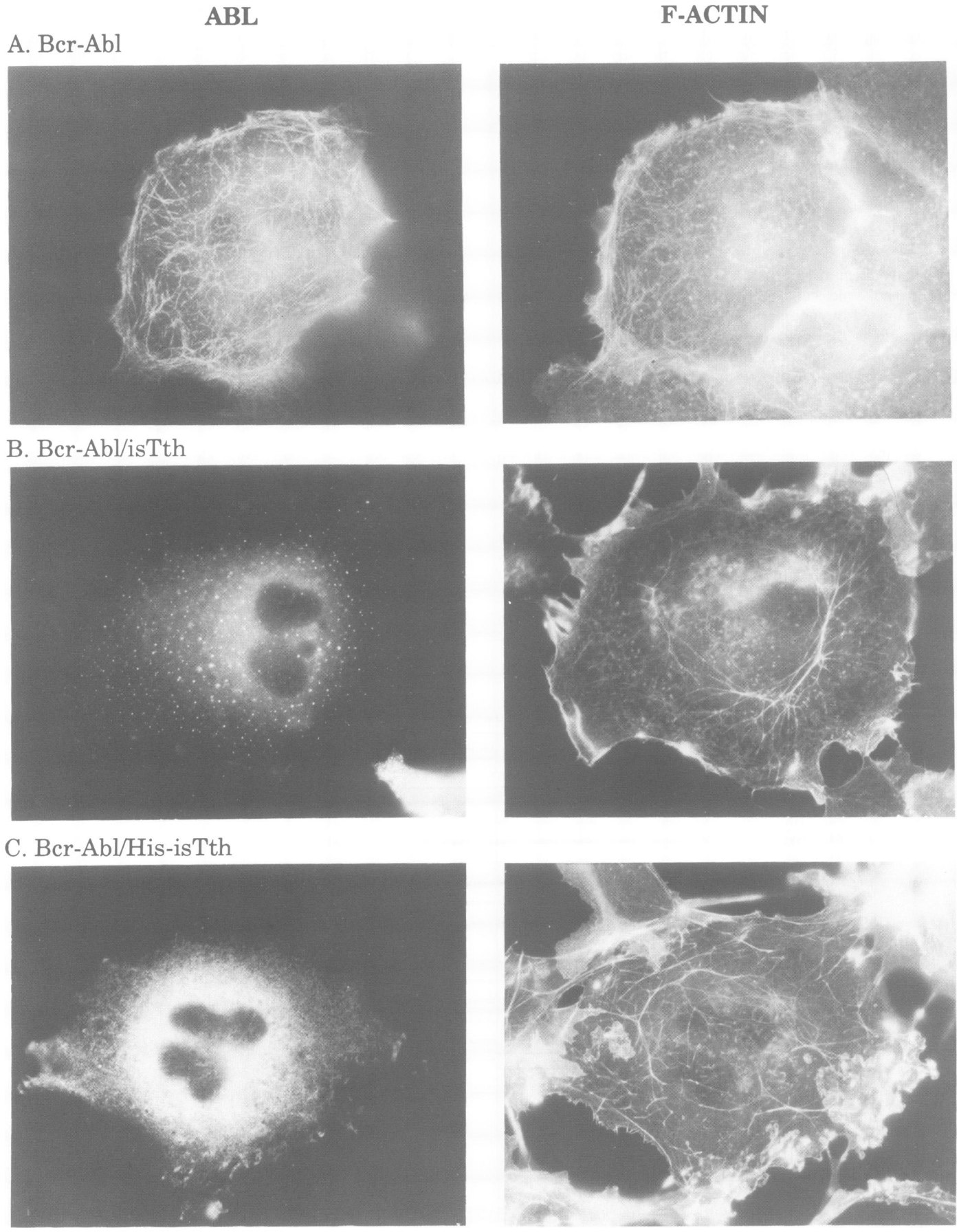
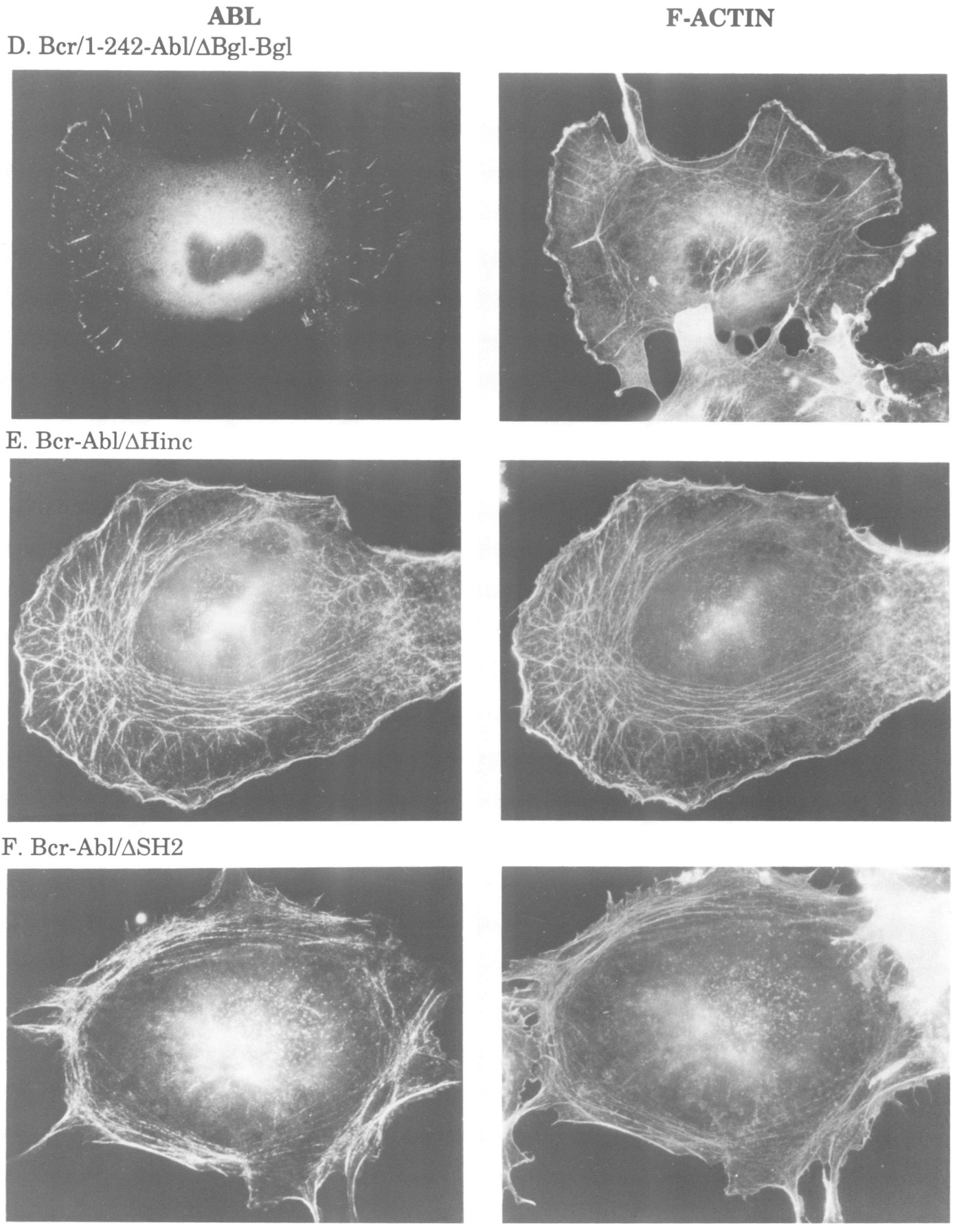
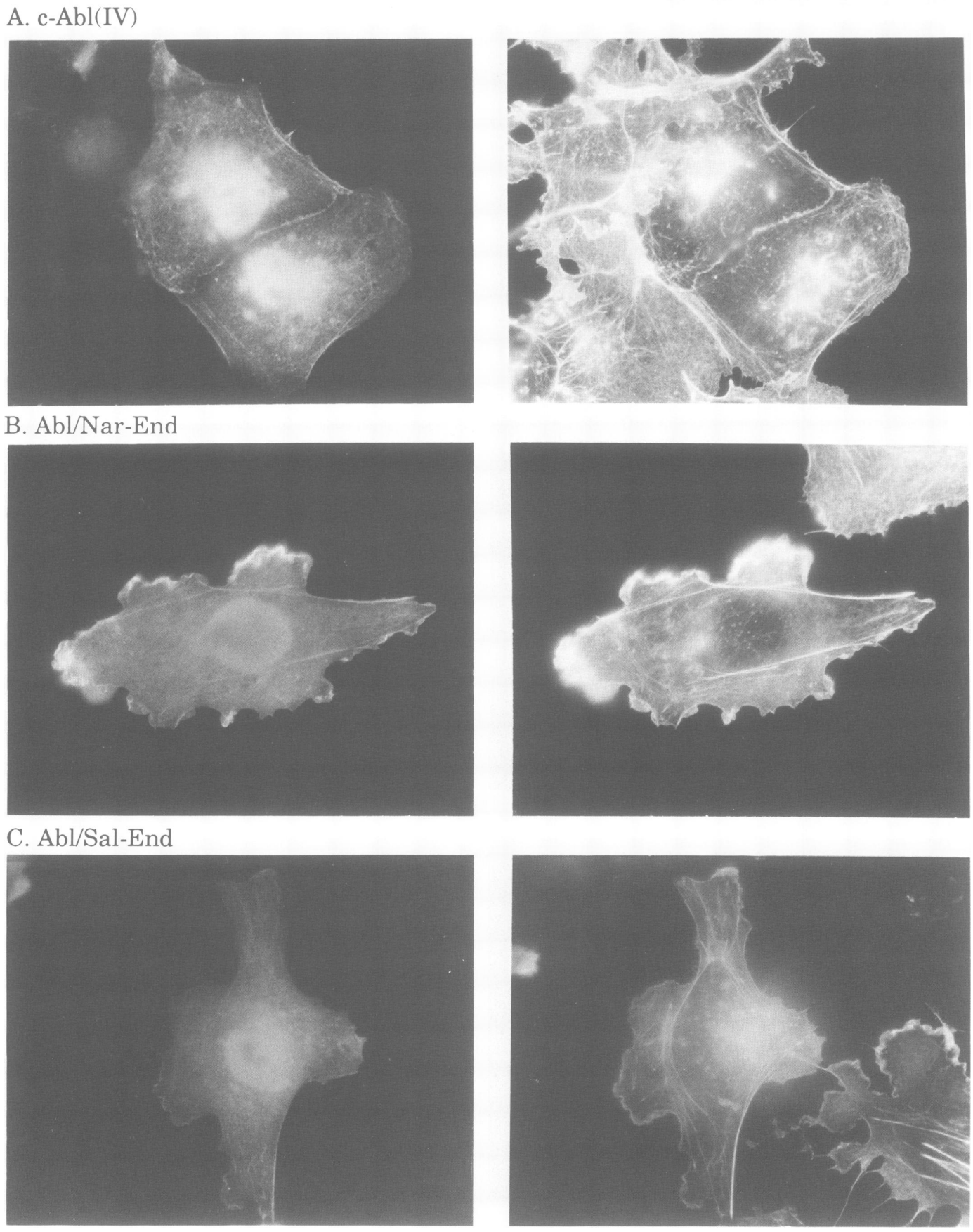


Fig. 3. Mapping of Abl sequences required for association with F-actin by double-label immunofluorescence. At 48 h after transfection with the indicated Bcr-Abl constructs, COS cells were prepared for immunofluorescence as described in Materials and methods. Primary antibody was anti-



Abl 8E9 (A–E) or anti-Abl CTN2 (F). Secondary antibody was rhodamine-conjugated goat anti-mouse (A–E) or goat anti-rabbit (F). Note that COS cells that were not transfected became stained with phalloidin but not with the anti-Abl antibody.


ABL
F-ACTIN
A. c-Abl(IV)
B. Abl/Nar-End
C. Abl/Sal-End

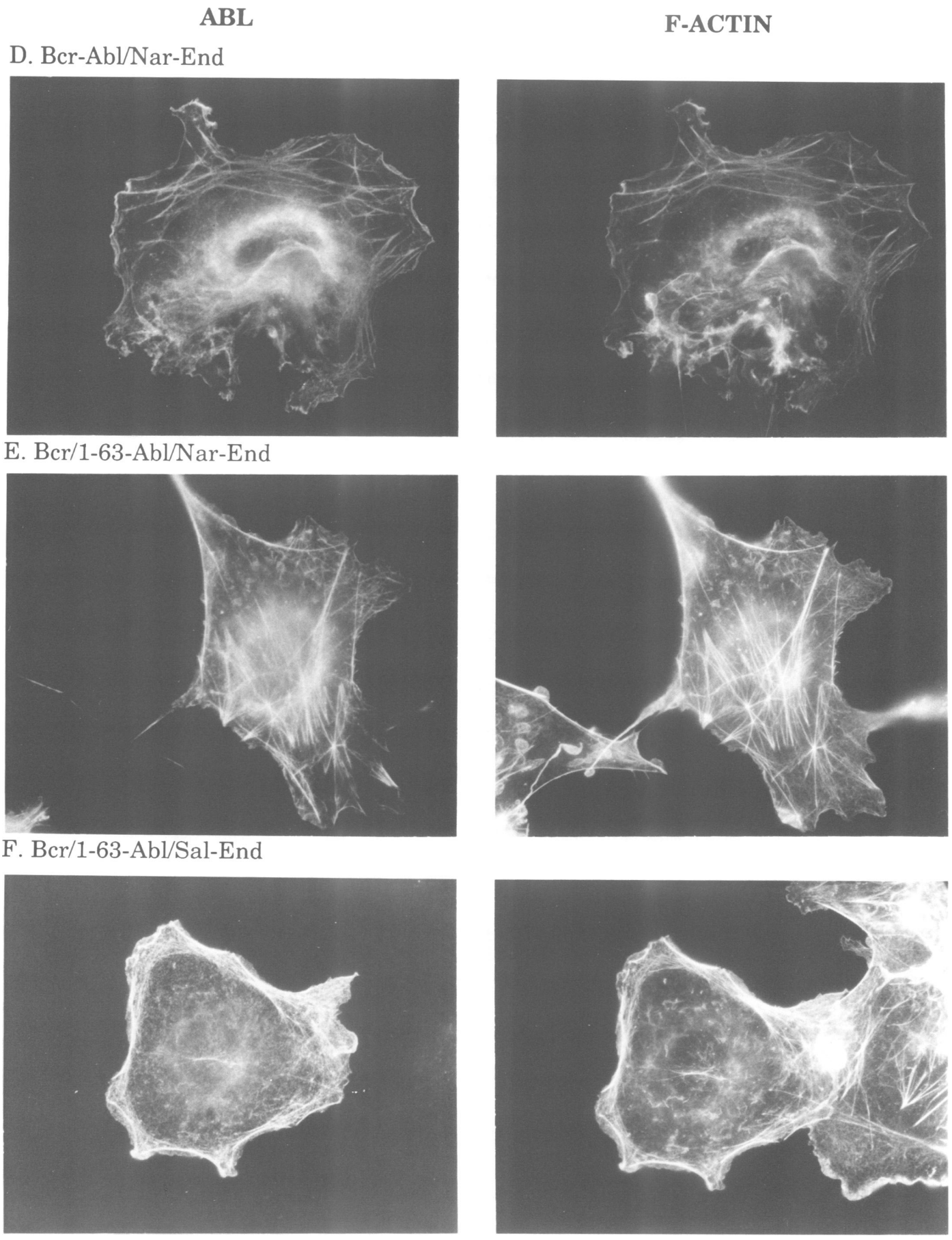


Fig. 4. Enhancement of F-actin association by Bcr sequences. At 48 h after transfection with the indicated constructs, COS cells were prepared for immunofluorescence and stained as described in Materials and methods. Primary antibody was anti-Abl CTN2. Secondary antibody was rhodamine-conjugated goat anti-rabbit. Compare panels D and E with panel B, or panel F with panel C to see enhancement by Bcr sequences.

also completely eliminated actin association (Figure 3B; Figure 2, isTth). These results showed that the C-terminal amino acids of Abl are critical to F-actin-binding.

In many cells, the actin-binding-defective Bcr-Abl proteins (ΔNar, ΔSal, ΔBgl, ΔTth and isTth) showed punctate staining in the cytoplasm (Figure 3B). These dots appeared to be at the lower surface of the cell, and in some cases colocalized with a punctate form of F-actin (Figure 3B). However, the majority of the dots did not contain detectable F-actin. A kinase-defective mutant that contained both the isTth mutation and a Lys → His point mutation in the c-Abl ATP-binding site did not associate with F-actin nor did it exhibit any punctate staining (Figure 3C; Figure 2, His-isTth). Thus, in addition to the kinase-independent association with actin filaments, Bcr-Abl also exhibited a kinase-dependent association with punctate structures which in some cases also contain F-actin.

Two internal deletions were made in the C-terminal segment. Deletion of c-Abl amino acids 712−934 had no detectable effect on actin association (Figure 2, ΔXho-Sal). Deletion of amino acids 321−1065 of c-Abl, which removes half of the kinase domain and all except the last 32 amino acids of the C-terminal segment, reduced but did not eliminate actin association (Figure 3D; Figure 2, Bcr/1-242-Abl/ΔBgl-Bgl). This deletion caused a qualitative change in the pattern of actin association. The majority of this mutant protein was localized diffusely in the cytoplasm (Figure 3D). However, some of it colocalized specifically with the ends of actin bundles that were anchored to the plasma membrane (Figure 3D). Together, these results show that actin association requires a domain located between amino acids 935 and 1097 of c-Abl. Moreover, amino acids 1066−1097 contain at least part of the actin-binding function.

The c-Abl protein contains a kinase-regulatory domain which includes the SH3 and 2 regions (Figure 2). Several other actin-binding proteins contain SH3 regions (Rodaway *et al.*, 1989). This has led to speculation that SH3 may be involved in actin-binding. SH2 regions are capable of forming stable complexes with phosphotyrosine-containing proteins (for reviews see Cantley *et al.*, 1991; Koch *et al.*, 1991). It was possible that association of Bcr-Abl with actin might be facilitated by binding of the SH2 region to a tyrosine-phosphorylated protein in actin filaments. To test these hypotheses, precise deletions were made in Bcr-Abl and the mutants were tested for actin-binding (Figure 2, ΔHinc and ΔSH2). Deletion of the SH3 or SH2 regions had no detectable effect on the degree of actin association (Figure 3E and F). Thus, SH3 and SH2 are not required for association of Bcr-Abl with actin filaments.

Association of C-terminal fragments of Abl with F-actin is enhanced by fusion to Bcr sequences

To determine if the C-terminal segment of c-Abl is sufficient for actin association, two C-terminal fragments containing amino acids 586−1097 and 934−1097 of c-Abl were expressed (Figure 2, Abl/Nar-End and Abl/Sal-End). Like the full-length c-Abl(IV) protein, the C-terminal fragments were localized in both the nucleus and the cytoplasm and a small fraction of the total protein colocalized with actin filaments (Figure 4, compare panel A with panels B and C). To determine if Bcr sequences could enhance their association with F-actin, hybrids were constructed in which Bcr amino acids 1−509 or 1−63 were fused to the Abl/Nar-End or Abl/Sal-End fragments (Figure 2: Bcr-Abl/Nar-End, Bcr/1-63-Abl/Nar-End, Bcr-Abl/Sal-End and Bcr/1-63-Abl/Sal-End). Comparison of the localization of the Bcr-Abl/Nar-End protein with that of the Abl/Nar-End protein showed that the Bcr/1-509 sequence led to exclusion of the protein from the nucleus and a dramatic increase in its association with F-actin (Figure 4D). The Bcr/1-63 sequence had a similar effect on the localization (Figure 4E). β-galactosidase sequences used as a control had no detectable effect on the localization of the Abl/Nar-End protein (Figure 2: βGal-Abl/Nar-End). In particular, they did not prevent the protein from entering the nucleus, suggesting that the Abl/Nar-End fragment contained a nuclear translocation signal distinct from the one located at amino acids 579−583 of c-Abl (Figure 2, NT; Van Etten *et al.*, 1989). Fusion of Bcr amino acids 1−509 or 1−63 to the Abl/Sal-

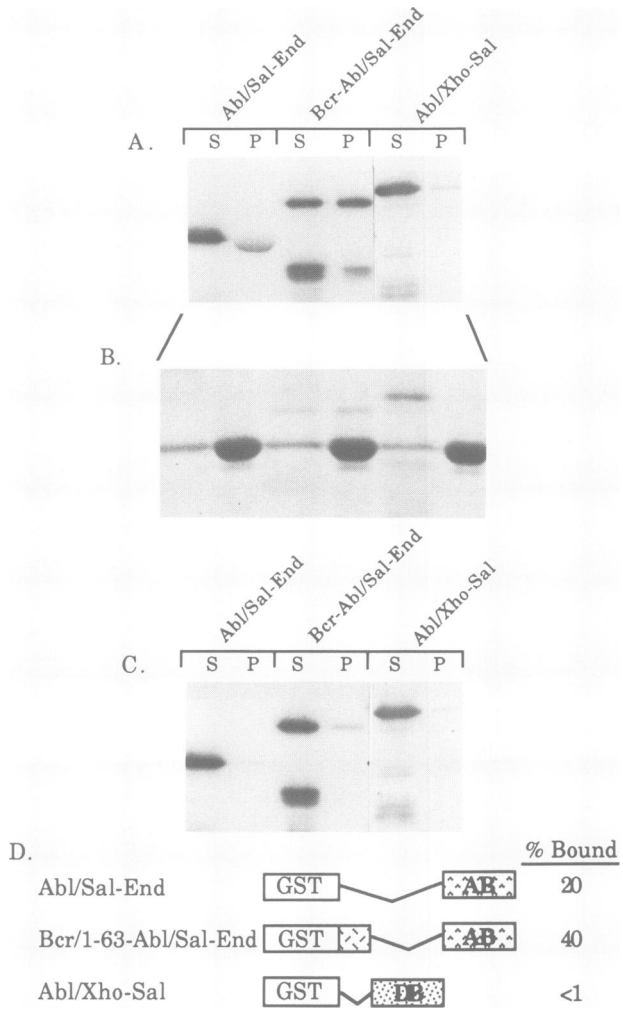


Fig. 5. Binding of Abl/Sal-End and Bcr/1-63-Abl/Sal-End proteins to F-actin *in vitro*. The indicated GST fusion proteins were incubated alone or with F-actin and sedimented as described in Materials and methods. Equal percentages of the supernatant (S) and pellet (P) fractions were separated on 10% polyacrylamide−SDS gels and immunoblotted with the anti-Abl antibody CTN2. (**A**) Immunoblot of GST fusion proteins sedimented with F-actin; (**B**) identical gel stained with Coomassie Blue to show distribution of actin; (**C**) immunoblot of GST fusion proteins sedimented without F-actin; (**D**) structures of GST fusion proteins and summary of results. AB: actin-binding domain. DB: DNA-binding domain. % Bound = % of GST protein in actin pellet (panel A, lanes P) minus % of GST protein in control pellet (panel C, lanes P).

End fragment also prevented nuclear translocation and enhanced its association with F-actin (Figure 4F). These results show that the Bcr/1-63 region has two effects on c-Abl localization: it prevents nuclear translocation and it specifically activates the actin-binding domain.

The c-Abl actin-binding domain binds to F-actin in vitro

Colocalization with F-actin *in vivo* does not prove that Bcr-Abl binds directly to F-actin. Therefore, a co-sedimentation assay was used to determine if Abl and Bcr-Abl proteins could bind to F-actin *in vitro*. The Abl/Sal-End and Bcr/1-63-Abl/Sal-End proteins were expressed in bacteria as glutathione-*S*-transferase (GST) fusion proteins (Figure 5D) and purified by affinity chromatography on glutathione−agarose beads. The fusion proteins were incubated alone or with purified rabbit muscle F-actin, layered over a 20% sucrose cushion and centrifuged to pellet the F-actin. The supernatants and pellets were separated on SDS−polyacrylamide gels and immunoblotted with the anti-Abl antibody CTN2 (Figure 5A and C) or stained with Coomassie blue (Figure 5B). The results were quantified by scanning the autoradiographs in Figure 5 (panels A and C) with a densitometer and are summarized in Figure 5D. Approximately 20% of the Abl/Sal-End protein and 40% of the Bcr-Abl/Sal-End protein cosedimented with the F-actin (Figure 5A and D). A GST protein containing the DNA-binding domain of c-Abl (amino acids 712−934) did not cosediment with F-actin in this assay (Figure 5: Abl/Xho-Sal). Therefore, cosedimentation with F-actin requires the presence of the Sal-End region. These results demonstrate that the C-terminal domain of c-Abl can bind weakly to F-actin and that fusion of Bcr sequences to this domain increases its affinity for F-actin. Thus, the ability of the proteins to bind F-actin *in vitro* correlates with their ability to colocalize with F-actin *in vivo*. However, the fact that less than half of the proteins cosedimented suggests that Abl may have a lower affinity for F-actin than most known actin-binding proteins.

Bcr-Abl proteins defective for actin association have reduced transforming activity

Bcr-Abl has been shown to cooperate with the *myc* oncogene to transform Rat-1 fibroblasts (Lugo and Witte, 1989). A Rat-1 cell line that overproduces the c-*myc* protein 5- to 10-fold was used to measure the transforming efficiencies of Bcr-Abl proteins. The cells were cotransfected with *bcr-abl* genes in retroviral expression vectors and the hygromycin (Hyg) resistance gene. In some experiments, a helper virus DNA clone (Moloney murine leukemia virus) was also co-transfected so that *bcr-abl* virus would be produced after transfection. The transfected cells were divided into three parts and assayed for focus formation, soft agar colony formation and Hyg-resistance. The transforming efficiencies were defined as the ratio of foci (or soft agar colonies) to Hyg-resistant colonies, and these ratios were normalized to that of the unmutated Bcr-Abl (Table I, last two columns).

A protein that contained only the c-Abl sequences (Figure 2, c-Abl/ΔPst), had no transforming activity (Table I, c-Abl/ΔPst). Therefore, as previously demonstrated by Muller *et al.* (1991), the Bcr sequences are absolutely required for transformation. The two mutants that were defective for actin-binding, Bcr-Abl/isTth and Bcr-Abl/ΔSal, had reduced transforming efficiencies. The isTth mutation caused a 5-fold decrease in focus formation and a 1.5- to 2-fold decrease in soft agar colony formation (Table I). The ΔSal mutation caused a 10-fold decrease in focus formation and a 2- to 4-fold decrease in soft agar colony formation (Table I). As a control, we tested a mutant that had a deletion of the c-Abl DNA-binding domain but which could still bind to actin (Figure 2, ΔXho-Sal). This mutation had no effect on the transforming efficiency (Table I). Thus, the DNA-binding function is not required for transformation. This is consistent with the fact that Bcr-Abl is excluded from the nucleus.

Table I. Transformation of Rat-1/myc cells by Bcr-Abl proteins

Helper virus DNA	Gene expressed	Soft agar/μg[a]	Foci/μg[b]	Hyg col/0.1 μg[c]	Soft agar/Hyg[d] (% of bcr-abl)	Foci/Hyg[d] (% of bcr-abl)
−	vector	2	0	153	5	0
	bcr-abl	37	24	138	100	100
	bcr-abl/isTth	18	5	123	55	23
	bcr-abl/ΔSal	10	3	152	25	11
	bcr-abl/ΔXho-Sal	44	31	134	123	133
	bcr-abl/ΔSH2	14	1	136	38	4
+	vector	4	0	262	5	0
	c-*abl*/ΔPst	9	0	251	11	0
	bcr-abl	75	191	228	100	100
	bcr-abl/isTth	65	40	283	70	17
	bcr-abl/ΔSal	37	15	250	45	7
	bcr-abl/ΔXho-Sal	69	213	222	94	114
	bcr-abl/ΔSH2	16	11	249	20	5

Rat-1/myc cells were cotransfected with 5 μg of *bcr-abl* plasmid in vector pSLX CMV, 0.5 μg of pRSV Hyg, and either 25 μg of salmon sperm DNA (− helper virus DNA) or 25 μg of a genomic clone of Mo-MuLV (+ helper virus DNA). 60 h after transfection, the cells were split 1:3. One-third were selected for hygromycin-resistance, one-third for focus formation and one-third for growth in soft agar medium. Foci and soft agar colonies were scored after 14 days. Each number is the average from two experiments.

[a]Number of soft agar colonies per microgram of *bcr-abl* plasmid.

[b]Number of foci per microgram of *bcr-abl* plasmid.

[c]Number of hygromycin-resistant colonies per 0.1 μg of pRSVHyg plasmid.

[d]Ratio of soft agar colonies (or foci) to hygromycin-resistant colonies expressed as a percentage of the ratio for the unmutated *bcr-abl* gene.

The SH2 domain was also found to be necessary for efficient transformation. Deletion of the SH2 domain from Bcr-Abl caused a 20-fold decrease in focus formation and a 2.5- to 5-fold decrease in soft agar colony formation (Table I: Bcr-Abl/ΔSH2). Therefore, both the actin-binding and SH2 domains contribute to transformation of Rat-1 cells by Bcr-Abl.

The expression of the Bcr-Abl proteins in soft agar colonies and foci was examined by immunoblotting. Most cell lines examined expressed proteins of the expected molecular weights (data not shown). However, in the presence of helper virus, cells transformed by the isTth mutant frequently expressed gene products that migrated faster than expected on SDS−polyacrylamide gels (data not shown). An altered gene product was also detected in one ΔSal-transformed cell line (data not shown). This suggested that rearrangements occurred in the isTth and ΔSal genes due to recombination with the helper virus, and that some of these mutants were selected for during the assay, possibly because they had higher transforming activity than isTth and ΔSal.

The reduced transforming efficiencies of the mutant proteins were not due to decreased stabilities or tyrosine kinase activities. When expressed in COS cells, the isTth, ΔSal and ΔXho-Sal proteins were expressed at similar steady-state levels and contained similar amounts of PTyr as the unmutated Bcr-Abl protein (data not shown). The unmutated Bcr-Abl, isTth, ΔSal and ΔXho-Sal proteins had half-lives of 8, 6.5, 4 and 8 h, respectively, in pulse−chase experiments (data not shown). These results were confirmed in Rat-1/myc cell lines expressing the proteins. In Rat-1 cells, the unmutated Bcr-Abl, isTth, ΔSal and ΔXho-Sal proteins had half-lives of ~6, 2, 8 and 8 h, respectively (data not shown). The relative levels of PTyr on the unmutated Bcr-Abl, isTth, ΔSal and ΔXho-Sal proteins were 1.0, 0.7, 1.8 and 2.2, respectively, determined by quantitative immunoblotting with antibodies for Abl and PTyr (data not shown). These results show that the reduced transforming efficiency of the actin-binding-defective mutants is unlikely to be due to decreased stability or autokinase activity.

Bcr-Abl proteins defective for actin-association have a reduced ability to abrogate IL-3 dependence in a lymphoid cell line

Bcr-Abl has also been shown to abrogate interleukin-3 (IL-3) dependence in the lymphoblastoid cell line Ba/F3 (Daley *et al.*, 1992). The mutant *bcr-abl* genes were introduced into Ba/F3 cells by electroporation and G418-resistant populations were isolated. IL-3 was then removed from the growth medium and the number of live cells in the cultures was monitored over a period of 3 weeks (Figure 6). All of the cultures were seeded at the same cell density to ensure that differences in growth were not due to differences in density. In the first 10 days, there was an ~10-fold decrease in cell number in all of the cultures, indicating that the majority of the transfected cells did not become IL-3-independent (Figure 6). After 14 days, the cells transfected with unmutated Bcr-Abl and ΔXho-Sal began to proliferate with a doubling time of 24 h (Figure 6). In contrast, growth of the cells transfected with the isTth and ΔSal mutants lagged behind by ~2 and 4 days, respectively (Figure 6). This indicates that fewer cells were made IL-3 independent by the isTth and ΔSal proteins. After the lag period, IL-3 independent cells in the isTth and ΔSal-transfected cultures

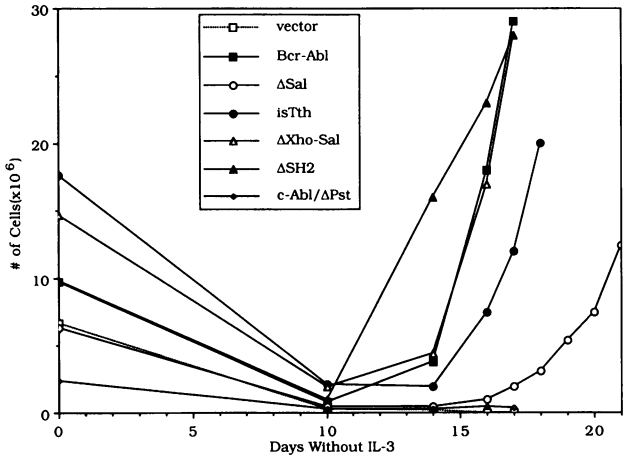


Fig. 6. Abrogation of IL-3 dependence in Ba/F3 cells by Bcr-Abl proteins. Ba/F3 cells were transfected with the indicated pSLXCMV constructs as described in Materials and methods. Stable transfectants were isolated by selection with G418. After 12 days of G418 selection, cells were transferred to growth medium lacking a source of IL-3. All cells were initially seeded at the same density. After the removal of IL-3, live cells were counted at the indicated times. Vector: cells transfected with the pSLXCMV vector; Bcr-Abl: cells transfected with the unmutated Bcr-Abl; ΔSal, isTth, ΔXho-Sal and ΔSH2: cells transfected with the mutant Bcr-Abl constructs (described in Figure 2); c-Abl/ΔPst: cells transfected with a construct containing only the Abl portion of Bcr-Abl.

grew with doubling times of 24 and 32 h, respectively. Therefore, the lag for the isTth culture was approximately two cell cycles and the lag for the ΔSal culture was approximately three cell cycles. This suggests that there was a 4-fold reduction in transforming efficiency for isTth and an 8-fold reduction for ΔSal, which is consistent with the results in Rat-1 cells (Table I). Moreover, in the isTth culture, only altered gene products similar in size to those observed in Rat-1 cells were detected by Western blotting (data not shown). These results show that the actin-binding function also contributes to the transformation of lymphoid cells by Bcr-Abl.

Surprisingly, the ΔSH2 mutant transformed Ba/F3 cells as efficiently as, if not better than, the unmutated Bcr-Abl (Figure 6). Therefore, the SH2 domain is not important for the abrogation of IL-3 dependence. A cell-type-specific requirement for SH2 has been observed previously for the *src* oncogene, where mutations in the SH2 domain interfere with transformation of mouse fibroblasts but not with transformation of chicken fibroblasts (Hirai and Varmus, 1990).

The results of Parmar *et al.* (1991) suggest that the actin-binding domain may also be important for transformation of primary lymphoid cells by the v-*abl* oncogene of Abelson murine leukemia virus. Although the C-terminal segment of v-*abl* is not required for transformation of fibroblasts, variants of v-*abl* that lack short portions of the C-terminal segment have a greatly reduced ability to transform primary lymphoid cells. The region that contributes to lymphoid transformation includes the actin-binding domain but not the DNA-binding domain (Parmar *et al.*, 1991).

Bcr-Abl transformed cells contain punctate, juxtanuclear F-actin aggregates in which the Bcr-Abl protein is localized

Rat-1/myc cells transformed by Bcr-Abl contained fewer actin stress fibers than the parental cell line (Figure 7,

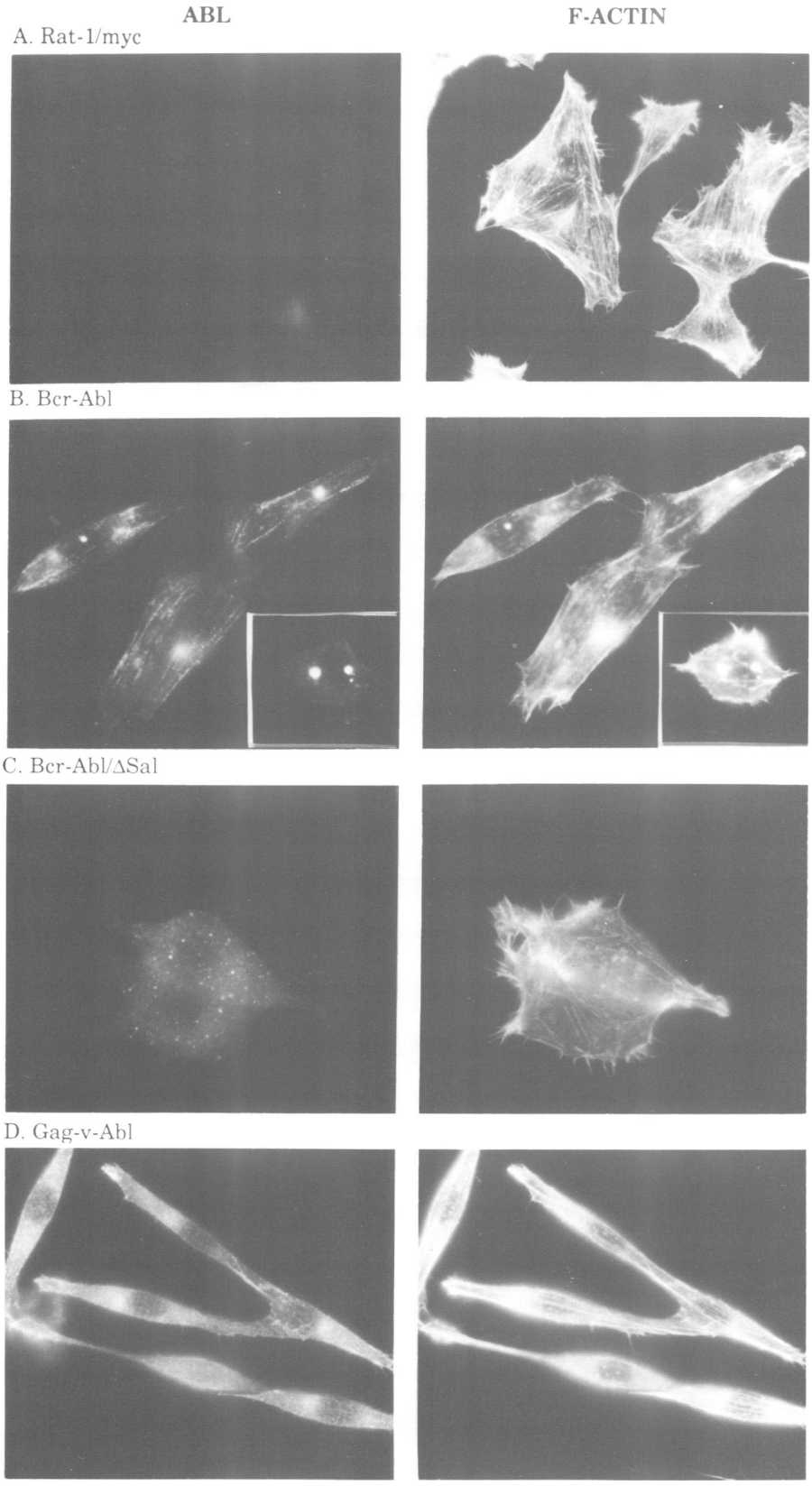


Fig. 7. Subcellular localizations of Bcr-Abl and Gag-v-Abl proteins in transformed Rat-1/myc cells. Untransfected Rat-1/myc cells or cell lines expressing the indicated proteins were prepared for immunofluorescence and stained as described in Materials and methods. Primary antibody was anti-Abl 8E9. Secondary antibody was rhodamine-conjugated goat anti-mouse. (**A**) Untransfected cells; (**B**) Bcr-Abl-transformed cells showing colocalization of the Bcr-Abl protein with stress fibers and punctate, cytoplasmic F-actin structures in flatter cells (main panel) and with large, juxtanuclear aggregates of F-actin in more rounded cells (inset); (**C**) Bcr-Abl/ΔSal-transformed cells showing punctate localization of the Bcr-Abl/ΔSal protein on the lower cell surface; (**D**) Gag-v-Abl-transformed cells showing diffuse cytoplasmic and plasma membrane localization of $p160^{gag\text{-}v\text{-}abl}$. Note the weak colocalization of $p160^{gag\text{-}v\text{-}abl}$ with stress fibers in some cells, similar to the pattern observed with c-Abl, and the absence of punctate F-actin aggregates.

compare panels A and B). However, the unmutated Bcr-Abl (Figure 7B) and ΔXho-Sal (data not shown) proteins colocalized with the remaining stress fibers. The staining of Bcr-Abl along stress fibers was punctate and Bcr-Abl appeared to be excluded from the F-actin in lamellipodia and filopodia at the leading edge of the cell (Figure 7B). This is similar to the pattern observed for Bcr-Abl in NIH3T3 cells (McWhirter and Wang, 1991) but differs from that observed in COS cells. Bcr-Abl can apparently bind to all forms of actin filaments when expressed at high levels in COS cells, but binds preferentially to stress fibers when expressed at lower levels in Rat-1 or 3T3 cells. In most of the Rat-1 cells, Bcr-Abl (Figure 7B) and ΔXho-Sal (data not shown) also colocalized with dot-like F-actin structures in the cytoplasm. These structures were usually located adjacent to the nucleus. In cells with a highly rounded morphology, the majority of the Bcr-Abl protein was often localized in one or two large, spherical, juxtanuclear F-actin aggregates (Figure 7B, inset). These structures were induced by Bcr-Abl as they were never observed in the parental Rat-1/myc cell line (Figure 7A) or in Rat-1/myc cells transformed by $p160^{gag\text{-}v\text{-}abl}$ (Figure 7D). Similar structures were also induced by Bcr-Abl in transformed Ba/F3 cells (data not shown).

As expected, the ΔSal (Figure 7C) and isTth (data not shown) proteins did not colocalize with actin stress fibers in Rat-1 cells. They exhibited a diffuse localization in the cytoplasm and a punctate localization both at the lower cell surface and in the cytoplasm (Figure 7C). The dots at the cell surface were similar to those observed in COS cells and appeared to contain little or no F-actin (compare Figure 7C with Figure 3B). The dots in the cytoplasm resembled those in Rat-1 cells expressing the unmutated Bcr-Abl protein: they colocalized with punctate F-actin and were often clustered in a juxtanuclear location (data not shown).

Discussion

An F-actin binding domain has been identified at the C-terminal end of the c-Abl protein. The affinity for F-actin is increased by the addition of Bcr sequences that are required for transformation by Bcr-Abl. The mechanism by which Bcr enhances the F-actin-binding function appears to involve an oligomerization domain located in the first 63 amino acids of Bcr (J.R.McWhirter, D.L.Galasso and J.Y.J.Wang, in preparation). Bcr-Abl proteins with mutations in the actin-binding domain are impaired in their ability to transform fibroblasts and lymphoid cells.

Sequence similarity of the C-terminal end of Abl to actin-crosslinking proteins

A consensus motif has been found in several actin-crosslinking proteins such as α-actinin and fimbrin (Adams *et al.*, 1991; Matsudaira, 1991). We have found that part of this motif occurs at the C-terminal end of members of the Abl family, including the mouse and human c-Abl proteins, the human Arg (Abl-related gene) protein, and the *Drosophila* Abl (D-Abl) protein (Figure 8). Two mutations that abolish actin-binding, ΔTth and isTth, disrupt the consensus motif (Figure 8). A 32 amino acid region (BglII-End) that contains the motif can colocalize with the ends of actin bundles (Figures 8 and 3D). These results show that the consensus motif is important for binding to F-actin. The weak similarity between c-Abl and the consensus motif may explain the relatively weak binding of c-Abl to F-actin observed both *in vivo* and *in vitro*. It may be that c-Abl has a lower affinity for F-actin than other actin-binding proteins because its function is to regulate the structure of the cytoskeleton as opposed to forming part of the structure (i.e. to crosslink filaments). By causing the c-Abl protein to form oligomers and increasing its affinity for F-actin, Bcr sequences may have the effect of converting c-Abl into an actin-crosslinking protein.

Secondary structure analysis of the c-Abl protein has suggested that the C-terminal segment consists of two distinct domains: a flexible, proline-rich domain (aa 467–967) and a rigid, globular domain (aa 967–1097) (Fainstein *et al.*, 1989). Within the Abl subfamily of tyrosine kinases, the globular domain is much more evolutionarily conserved than the proline-rich domain. The entire C-terminal segment of the human c-Abl protein is only 74, 29 and 16% identical to the mouse c-Abl, human Arg and D-Abl proteins, respectively. In contrast, the last 130 amino acids are 96% identical between the human and mouse proteins, the last 60 amino acids are 56% identical between the human c-Abl and Arg proteins, and the last 50 amino acids are 32% identical (and 56% similar) between the human c-Abl and D-Abl proteins (Fainstein *et al.*, 1989; Henkemeyer *et al.*, 1988; Kruh *et al.*, 1990). Our results show that this conserved region is an actin-binding domain. Therefore, all members of the Abl subfamily should have the ability to associate with F-actin.

Implications of actin-binding for normal c-Abl function

The evolutionary conservation of the actin-binding domain suggests that it may be important for normal c-Abl function. Genetic analysis of *abl* in flies and mice has revealed that the C-terminal segment is required for the normal

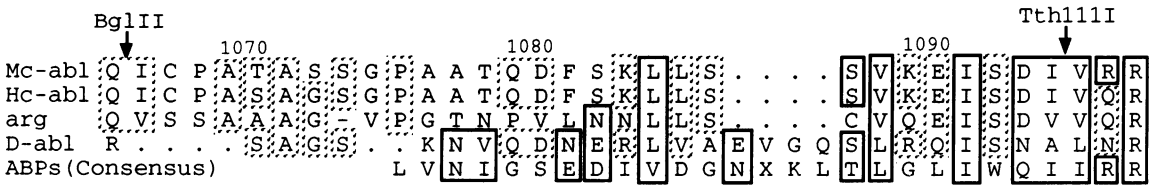


Fig. 8. Sequence similarity between the c-Abl actin-binding domain and other actin-binding proteins. Amino acids 1065–1097 of murine c-Abl and the homologous regions from the human c-Abl (Hc-abl), human arg (arg), and *Drosophila* Abl (D-abl) proteins were aligned by eye with a 27 amino acid consensus motif (ABP consensus) found in the actin-crosslinking proteins α-actinin, fimbrin, Sac6p, L-plastin, β-spectrin, dystrophin, ABP-120 and ABP-280 (Adams *et al.*, 1991; Matsudaira, 1991). BglII and Tth111I indicate the positions of deletion or insertion mutations that affect actin-binding (see Figures 2 and 3). Boxes with solid lines: Abl residues with similarity to the actin-binding consensus motif. Boxes with dashed lines: residues conserved in Abl subfamily. Dots represent gaps in the alignment.

biological function of the Abl tyrosine kinase. In flies, the C-terminal segment of the *Drosophila* Abl (D-Abl) protein is necessary for rescue of the mutant phenotype and for proper localization of the protein in axon bundles of the embryonic central nervous system (Henkemeyer *et al.*, 1990). In mice, gene knockout experiments have shown that deletion of the C-terminal 385 amino acids from c-Abl results in the same phenotype as a disruption of the tyrosine kinase domain: neonatal lethality and lymphopenia (Schwartzberg *et al.*, 1991; Tybulewicz *et al.*, 1991). It is intriguing that this 385 amino acid region of c-Abl also contains a sequence-specific DNA-binding domain (Figure 2; Kipreos and Wang, 1992; J.Y.J.Wang, unpublished data). The presence of both actin-binding and DNA-binding domains in c-Abl implies that the protein has both a cytoplasmic and a nuclear function. Moreover, it suggests that the protein may be capable of relaying a signal from the cytoskeleton to the genome in a single step. For example, an extracellular signal could cause the c-Abl protein to become translocated from the actin cytoskeleton to the nucleus where it could regulate the expression of specific genes.

Role of actin-association in transformation by Bcr-Abl

The role of actin-association in transformation by Bcr-Abl may be analogous to that of *N*-myristoylation in transformation by the v-Abl and v-Src oncoproteins. Efficient transformation may require that the tyrosine kinase be concentrated at the locations of critical substrates in the membrane or actin cytoskeleton. Alternatively, the kinase may have to form complexes with enzymes in the cytoplasm (i.e. phosphatidylinositol-3-kinase, Varticovski *et al.*, 1991) and bring them to their substrates in the plasma membrane or actin cytoskeleton. For example, IL-3 has been shown to stimulate hydrolysis of phosphotidylcholine to form diacylglycerol (DAG), which in turn activates protein kinase C (Ruggerio *et al.* 1991). In order to abrogate IL-3 dependence in Ba/F3 cells, Bcr-Abl may need to be targeted to actin filaments so that it can stimulate the formation of DAG. The idea that actin-binding is required for efficient interaction of Bcr-Abl with certain substrates is supported by the observation that a 41 kDa protein contains a much lower level of phosphotyrosine in cells expressing the non-actin-binding Bcr-Abl proteins (ΔSal and isTth) than in cells expressing the actin-binding Bcr-Abl proteins (data not shown). It is possible that there are other substrates, which we were unable to detect, whose phosphorylation contributes to transformation and is dependent on the actin-binding function of Bcr-Abl.

The localization of Bcr-Abl proteins and F-actin in transformed Rat-1 and Ba/F3 cells suggests that Bcr-Abl can induce a redistribution of F-actin from stress fibers or the cortical cytoskeleton into punctate, juxtanuclear aggregates (Figure 7). This process may interfere with normal signal transduction pathways by disrupting interactions between the actin cytoskeleton and growth factor or cell adhesion receptors in the plasma membrane. However, it is not yet known if these juxtanuclear F-actin aggregates are induced by Bcr-Abl in Ph^1-positive human leukemias.

Recent evidence suggests that the primary defect in CML may be discordant maturation, rather than unregulated proliferation, of Ph^1-positive myeloid progenitor cells (Clarkson and Strife, 1991). The proliferation and differentiation of hematopoietic cells is regulated by both cytokine receptors and cell adhesion receptors (reviewed by Kincade, 1990, and Springer, 1990). Whereas CML progenitor cells appear to respond normally to cytokines, some evidence suggests that they have defects in cell adhesion (Gordon *et al.* 1987; Guba and Emerson, 1991). For example, they do not express the cytoadhesion molecule LFA-3, which may be required to suppress their proliferation (Guba and Emerson, 1991). In long-term bone marrow cultures, excessive production of granulocyte–macrophage progenitors can occur when normal primitive progenitors are not allowed to interact directly with the stromal layer (Verfaillie, 1992). Therefore, CML could be caused by an inability of CML progenitors to interact directly with stromal cells in the bone marrow. Thus, $p210^{bcr\text{-}abl}$ may act not by stimulating mitogenesis but rather by interfering with cell adhesion signals that suppress growth or differentiation. Localization of p210 on actin filaments may allow it to interact directly with adhesion receptors or with proteins that link them to the cytoskeleton.

Materials and methods

Cell culture

COS cells were grown in Dulbecco's modified Eagle's medium (DME) supplemented with 10% calf serum and P/S (200 U/ml penicillin and 200 μg/ml streptomycin). Rat-1/myc cells were grown in DME supplemented with 10% fetal calf serum and P/S. K562 cells (Lozzio and Lozzio, 1975) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and P/S. Ba/F3 cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, P/S, and 10% WEHI-3B conditioned medium as a source of IL-3.

Plasmid construction

For expression in COS cells, mutants were constructed in the SV40 expression vectors pSVL or pcD (McWhirter and Wang, 1991; Okayama and Berg, 1983). Plasmids pSVL c-Abl(IV), c-Abl/ΔPst, Bcr-Abl and Bcr-Abl/ΔNar have been described previously (Wang, 1988; McWhirter and Wang, 1991). All deletion, insertion and fusion proteins were constructed using standard recombinant DNA methods, except for Bcr-Abl/ΔSH2, which was made using the polymerase chain reaction (W.M.Franz and J.Y.J.Wang, unpublished data).

The pSVLBcr-Abl/ΔSal, ΔBgl and ΔTth plasmids contain frameshift mutations at the *Sal*I, *Bgl*II or *Tth*111I restriction sites in the *abl* sequence, respectively. pSVLBcr-Abl/isTth was made by cutting pSVLBcr-Abl with *Tth*111I, blunting the ends with the Klenow fragment of DNA polymerase, fitting the ends with an 8 bp *Pvu*I linker, and religating. pSVLBcr-Abl/ΔXho-Sal is deleted from the *Xho*I site to the *Sal*I site in *abl* and joined by a *Pvu*I linker. pcDBcr/1-242-Abl/ΔBgl-Bgl is deleted from the *Bgl*II site in the *abl* tyrosine kinase domain to the *Bgl*II site in the C-terminal segment. pcDBcr-Abl/ΔHinc is deleted from the 5′ end of c-*abl* to the *Hin*cII site at the end of the SH3 domain and joined to the indicated Bcr sequences by an *Xba*I linker.

pSVLAbl/Nar-End and Abl/Sal-End contain codons for Met-Gly-Arg-Leu-Glu-Leu-Lys-Leu or Met-Gly-Arg fused to the 5′ ends of the *Nar*I–*Hin*dIII or *Sal*I–*Hin*dII restriction fragments of c-*abl*, respectively. The fused codons were derived from the multiple cloning site of pGEX-KG (Guan and Dixon, 1991). pcDBcr-Abl/Nar-End and Bcr/1-63-Abl/Nar-End contain the *Nar*I–*Bam*HI restriction fragment of c-*abl* joined to the indicated Bcr sequences by an *Eco*RI linker. pcDβGal-Abl/Nar-End contains the *Hin*dIII–*Eco*RI fragment of pCH110 (Pharmacia), which contains codons 1–1059 of the *lacZ* gene, joined to the *Nar*I–*Bam*HI fragment of c-*abl* by an *Eco*RI linker. pSVLBcr-Abl/Sal-End and Bcr/1-63-Abl/Sal-End contain the *Sal*I–*Hin*dIII restriction fragment of c-*abl* joined to the indicated Bcr sequences by an *Xba*I linker.

For expression in *Escherichia coli*, the Abl/Sal-End and Bcr/1-63-Abl/Sal-End genes were inserted into the multiple cloning site of the vector pGEX-KG, which allowed them to be produced as GST fusion proteins (Guan and Dixon, 1991).

Indirect immunofluorescence microscopy

COS cells were transfected as previously described (McWhirter and Wang, 1991). COS and Rat-1/myc cells were plated onto polylysine-coated cover

slips and grown overnight before preparing them for immunofluorescence. Cytochalasin B-treated or untreated K562 cells were seeded onto polylysine-coated cover slips and incubated for 10 min at room temperature before preparation. Cells were prepared for double-label immunofluorescence by fixation with formaldehyde and permeabilization with Triton X-100 as previously described (McWhirter and Wang, 1991). Rat-1/myc and K562 cells were blocked with 5% normal goat serum (NGS) for 15 min before staining and all antibody solutions contained 2% NGS. Primary antibody was monoclonal anti-Abl 8E9 (20 μg/ml; McWhirter and Wang, 1991) or affinity-purified polyclonal anti-Abl CTN2 (10 μg/ml). Secondary antibody was rhodamine-conjugated goat anti-mouse (10 μg/ml) or rhodamine-conjugated goat anti-rabbit (10 μg/ml). In all experiments shown, fluorescein isothiocyanate (FITC)-conjugated phalloidin (Molecular Probes) was added with the secondary antibody to visualize F-actin. However, in experiments using K562 and Rat-1 cells, cells stained with the anti-Abl antibody alone were included as a control. Cells were photographed as previously described (McWhirter and Wang, 1991).

F-actin cosedimentation

GST fusion proteins were produced in *E.coli* using the pGEX-KG vector and purified with glutathione–Sepharose beads (Guan and Dixon, 1991). Rabbit muscle F-actin was purified as described and was a gift from James A.Spudich (Pardee and Spudich, 1982). The GST fusion protein and rabbit muscle F-actin were mixed to final concentrations of 1 μM and 10 μM, respectively, in PKM buffer (20 mM PIPES, pH 6.8, 100 mM KCl, 2 mM $MgCl_2$, 0.2 mM ATP) plus 100 μg/ml BSA. The final volume was 0.2 ml. The mixture was incubated for 1 h at room temperature. The proteins were then layered over a 0.5 ml 20% sucrose cushion containing PKM buffer. Centrifugation was at 40 000 r.p.m. in a Beckman Ty65 rotor for 2 h at 4°C. The supernatant and pellet fractions were boiled in SDS sample buffer, separated on 10% polyacrylamide–SDS gels and immunoblotted with CTN2 antibody to detect Abl or stained with Coomassie Blue to detect actin. The percentage of GST fusion protein in the F-actin pellet fraction was determined by scanning the autoradiographs with a densitometer.

Rat-1 transformation assay

Bcr-abl genes were inserted into the *Bam*HI cloning site of the retroviral expression vector pSLX CMV (Scharfmann *et al.*, 1991). This allowed the *bcr-abl* genes to be expressed from the internal intermediate early cytomegalovirus (CMV) promoter. Rat-1/myc cells were a gift from Dr Robert N.Eisenman. They were made by infecting Rat-1 cells with a retroviral vector expressing the human c-*myc* gene from its long terminal repeat promoter and the neomycin-resistance gene from an interval SV40 early promoter. Infected cells were isolated by selection with G418. Rat-1/myc cells were cotransfected using the calcium phosphate coprecipitation method with 5 μg of pSLX CMV plasmid, 0.5 μg of a marker for Hyg-resistance (pRSV Hyg) and 25 μg of either salmon sperm DNA or pZAP. pZAP is a genomic clone of a replication-competent Moloney murine leukemia virus (McWhirter and Wang, 1991). Sixty hours after transfection, the cells were split into three parts and selected for focus formation, colony formation in soft agar medium or Hyg-resistance. Colony formation in soft agar was measured by resuspending the cells in 4 ml of medium plus 0.3% Noble agar and plating them in two 6 cm dishes containing a bottom layer of medium plus 0.6% Noble agar. Foci and soft agar colonies were scored after 14–15 days of selection. Hyg-resistant colonies were scored after 12 days of selection.

Ba/F3 transformation assay

Ba/F3 cells were electroporated using a BTX Transfector 300 (BTX, Inc., San Diego). 6×10^6 cells were washed twice with cold phosphate-buffered saline (PBS) and resuspended in 0.4 ml PBS containing 25 μg of *bcr-abl* construct in the vector pSLXCMV. The cells were pulsed once (1000 μF, 600 V/cm), kept on ice for 10 min and transferred to growth medium. Two days later, the cells were selected with G418. The G418-resistant populations were washed twice with PBS and then resuspended at 10^6 cells per ml in medium lacking a source of IL-3. At various time points, the number of live cells in each culture was determined using trypan blue stain and a hemocytometer.

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