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The COOH Terminus of the c-Abl Tyrosine Kinase Contains Distinct F- and G-Actin Binding Domains with Bundling Activity

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Abstract. The myristoylated form of c-Abl protein, as well as the P210$^{bcr/abl}$ protein, have been shown by indirect immunofluorescence to associate with F-actin stress fibers in fibroblasts. Analysis of deletion mutants of c-Abl stably expressed in fibroblasts maps the domain responsible for this interaction to the extreme COOH-terminus of Abl. This domain mediates the association of a heterologous protein with F-actin filaments after microinjection into NIH 3T3 cells, and directly binds to F-actin in a cosedimentation assay. Microinjection and cosedimentation assays localize the actin-binding domain to a 58 amino acid region, including a charged motif at the extreme COOH-terminus that is important for efficient binding. F-actin binding by Abl is calcium independent, and Abl competes with gelsolin for binding to F-actin. In addition to the F-actin binding domain, the COOH-terminus of Abl contains a proline-rich region that mediates binding and sequestration of G-actin, and the Abl F- and G-actin binding domains cooperate to bundle F-actin filaments in vitro. The COOH terminus of Abl thus confers several novel localizing functions upon the protein, including actin binding, nuclear localization, and DNA binding. Abl may modify and receive signals from the F-actin cytoskeleton in vivo, and is an ideal candidate to mediate signal transduction from the cell surface and cytoskeleton to the nucleus.

It has long been appreciated that there is an intimate connection between the cytoskeleton, cell growth, morphology, and the process of transformation. In the transformed state, fibroblasts and epithelial cells exhibit several morphological alterations including increased membrane ruffling, increased cell motility, increased saturation density, and loss of contact inhibition. This is accompanied by a dramatic reorganization of elements of the cytoskeleton, with loss of F-actin stress fibers, depolymerization of F-actin, and loss of focal adhesions. These alterations are associated with changes in cellular actin-binding proteins, such as the actin severing protein gelsolin (Vandekerckhove et al., 1990).

Transmembrane tyrosine kinases, such as the PDGF receptor, indirectly influence the response of the cytoskeleton to growth factor stimulation by activating members of the Ras family of small G proteins. p21^{rho} has been shown to induce formation of new stress fibers and focal adhesions (Ridley and Hall, 1992), while the related *ras* family member p21^{rac} serves to induce membrane ruffling and reorganization of the cortical actin network (Ridley et al., 1992), although the precise effectors of these G proteins are not known. Similar signal transduction pathways, leading from receptor tyrosine kinase activation to G proteins, have been implicated by genetic studies of *Caenorhabditis elegans* vulval development (Horvitz and Sternberg, 1991) and *Drosophila* retinal development (Simon et al., 1993).

Non-receptor tyrosine kinases, typified by the *src* gene product, have been shown to interact directly with the cytoskeleton. v-Src protein induces a dramatic reorganization of the cytoskeleton of chick embryo fibroblasts, an effect which occurs after temperature shift with a temperature-sensitive allele of v-Src. v-Src is found in focal adhesions (Rohrschneider, 1980) and a fraction of the protein is membrane associated, the latter interaction directed by the myristate group and NH_2-terminal amino acid sequences (Krueger et al., 1982; Kaplan et al., 1990). Several components of adhesion plaques are tyrosine phosphorylated in v-Src–transformed cells, although none of the substrates has been shown to be essential for transformation (Kamps et al., 1986). However, tyrosine phosphorylation of integrins in adhesion plaques of normal fibroblasts has been demonstrated (Hynes, 1992), and the effect of tyrosine phosphatase inhibitors on cell morphology strongly suggests an impor-

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tant role for tyrosine phosphorylation in cell adhesion and morphology (Klarlund, 1985).

Abl is a non-receptor protein-tyrosine kinase whose large COOH-terminal domain distinguishes it from members of the *src* family. Activated forms of Abl, typified by the viral transforming protein p160$^{gag/v\text{-}abl}$, transform both fibroblasts and hematopoietic cells, and are localized to the cytoplasm, plasma membrane, and focal adhesions, similarly to Src (Rohrschneider and Najita, 1984; Van Etten et al., 1989). Another activated form of Abl, the P210$^{bcr/abl}$ protein of human chronic myelogenous leukemia, is also localized to the cytoplasm, and is associated with F-actin microfilaments in fibroblasts, hematopoietic cells, and epithelial cells (Wetzler et al., 1993; Daley et al., 1992; McWhirter and Wang, 1991; McWhirter and Wang, 1993). The normal cellular Abl protein, c-Abl, is present in two forms which differ at their extreme NH_2 termini. The type IV/Ib protein is myristoylated on the NH_2-terminal glycine, while the type I/Ia protein is not. Neither protein can transform fibroblasts or hematopoietic cells, even upon overexpression (Jackson and Baltimore, 1989). The subcellular localization of the type IV c-Abl protein was determined in NIH 3T3 cells overexpressing the protein, and it was found to be largely nuclear, although appreciable amounts were found associated with the plasma membrane and in the cytoplasm (Van Etten et al., 1989). In addition, there was a prominent association of c-Abl with filamentous cytoplasmic structures resembling F-actin stress fibers.

Here we show that these structures indeed represent F-actin microfilaments, and map the domain of Abl responsible for this interaction to the extreme COOH terminus of Abl. This domain binds directly to F-actin in vitro. A subregion of this domain, distinct from the F-actin binding site, binds to the monomeric form of actin, G-actin. Together, the Abl F- and G-actin binding domains mediate bundling of F-actin in vitro.

Materials and Methods

Abl Expression Constructs and Mutants

For stable expression of Abl proteins in NIH 3T3 cells, the retroviral expression vector pPL was used (Jackson and Baltimore, 1989). NIH 3T3 cell lines stably expressing mutant Abl proteins were generated by calcium phosphate co-transfection with pSV2*neo*, and screening clones for expression by indirect immunofluorescence. The expression of the mutant proteins was confirmed in all cases by immunoblotting of cell extracts with anti-Abl antisera (data not shown). The construction and expression of pPLcIV, pPLcIVΔXB, and pPLcIVΔPH was previously described (Jackson and Baltimore, 1989). The mutant pPLcIVQ5 was generated by purifying the 4.1-kb EcoRI-HindIII fragment from pJ3cIVQ5 (Jackson and Baltimore, 1989) and ligating into the polylinker site of pPL. pPLcIV K290M was generated by site-specific mutagenesis utilizing the *dut/ung* phagemid system (Bio-Rad Laboratories, Beverly, MA), as described (Mayer et al., 1992). pPLcIV K290MΔSal, pPLcIV K290MΔBgl, and pPLcIV K290MΔTth were generated by digestion of pPLcIV K290M with SalI, BglI, or Tth111-I, respectively. A synthetic oligonucleotide containing an in-frame termination codon was ligated to the SalI-digested pPLcIV K290M DNA, truncating the COOH-terminal 160 amino acids of Abl. BglII-digested pPLcIV K290M DNA was blunted with the Klenow fragment of *Escherichia coli* DNA polymerase I, ligated to a 14-mer SpeI linker containing termination codons in all three reading frames, digested with SpeI and self-ligated. Tth111-I-digested pPLcIV K290M was blunted with Klenow fragment, ligated to BclI linkers, digested with BclI and self-ligated. The insertion of the BclI linker results in the generation of a TGA termination codon after isoleucine at position 1139, truncating the three COOH-terminal amino acids of Abl. All mutations were confirmed by DNA sequencing.

GST-Abl Fusion Proteins and Gelsolin S2-3 Domain

Portions of the Abl sequence, including the pEX5 COOH-terminal sequence, were expressed in *E. coli* as fusion proteins with the *Schistosoma japonicum* glutathione S-transferase (GST)[1] using the Glutagene system (Pharmacia, Piscataway, NJ) and purified by affinity chromatography on glutathione-agarose beads (Molecular Probes, Eugene, OR) as described (Van Etten et al., 1989). For production of NH_2-terminal deleted fusion proteins ΔFok, ΔSca, ΔHinf, and ΔBgl, the 1.1-kb SalI-HindIII insert of pEX5/pGEX-1 was digested with FokI, ScaI, HinfI, or BglII, and restriction fragments of 530, 916, 806, or 710 bp isolated, respectively. The FokI, ScaI, and HinfI fragments were blunted with Klenow fragment and cloned into the SmaI site of the appropriate pGEX vector, while the BglII fragment was cloned into the BamHI site of pGEX-3X. The COOH-terminal deleted fusion protein ΔSca(C) was produced by cloning the 205-bp BamHI/ScaI fragment from pEX5/pGEX-1 back into pGEX-1. The junction of the GST-Abl reading frame was verified in each case by DNA sequencing, and chimeric nature of the GST-Abl fusion proteins confirmed by immunoblotting bacterial cell extracts with anti-pEX5 antisera (data not shown). The GST-gelsolin fusion protein was generated by cloning the cDNA of the gelsolin S2-3 domain (kind gift of Dr. M. Way, Whitehead Institute, Cambridge, MA) into the pGEX-2T vector and purifying the 54-kD fusion protein on glutathione-agarose beads. After elution from the beads, fusion proteins were desalted by chromatography on Sephadex G-25 in 50 mM NH_4HCO_3, pH 7.8, followed by lyophilization. In general, final fusion protein preparations were 80–95% full-length protein, with small amounts of shorter species which did not interfere with the pelleting or microinjection assays.

Where indicated, fusion proteins expressed in the pGEX-2T vector and containing the thrombin cleavage site were cleaved by incubation with 1.0% (wt/wt) human thrombin (Calbiochem-Novabiochem Corp., La Jolla, CA) as described (Mayer et al., 1992; Smith and Corcoran, 1992) for 2 h at room temperature in the presence of 2.5 mM $CaCl_2$. Free GST and uncleaved fusion protein was removed by adsorption with glutathione-agarose beads, and the purified Abl protein was desalted by chromatography on Sephadex G-25 and concentrated by lyophilization.

The gelsolin S2-3 domain, containing the F-actin binding domain, was expressed as a 28-kD protein in *E. coli* using the pET vector system and BL21(DE3) host strain (Studier et al., 1990) (kind gift of Dr. M. Way, Whitehead Institute) essentially as described (Way et al., 1992). The final preparation was greater than 95% intact S2-3 protein on SDS-PAGE, and copelleted with F-actin with an estimated binding constant of about 1–5 μM (data not shown), consistent with previous observations (Way et al., 1992).

Cell Culture and Microinjection

NIH 3T3 cell lines were maintained in DME with 10% calf serum at 37°C in a humidified 5% CO_2 atmosphere. Cells were plated onto acid-washed glass coverslips at concentrations of 0.5–1.0 × 10^4 cells/cm^2 48 h before injection. Fusion proteins were resuspended in 1 mM Tris-acetate (pH 7.0) at 2.5–5.0 mg/ml, and introduced into cells by microinjection as described (Graessman et al., 1980) using a Zeiss Axiophot inverted phase contrast microscope. For each fusion protein, 30–50 cells were injected, and the average injection volume was about 2–5% of cell volume. 1 h after injection, cells were fixed and extracted with 4% paraformaldehyde (EM Sciences, Gibbstown, NJ) in 1× PHEM buffer, pH 6.1, with 0.1% Triton X-100 for 5 min at 37°C as described (Schliwa and van Blerkom, 1981). Detection of fusion protein was with affinity-purified rabbit antisera directed against GST used at 2 μg/ml, while secondary antibody was donkey anti-rabbit IgG, Texas red-conjugated (Jackson ImmunoResearch Labs Inc., West Grove, PA), at 15 μg/ml. Detection of F-actin was with FITC-conjugated phalloidin (Molecular Probes) used at a concentration of 6.7 U/ml (~0.22 μM) in PBS. After washing, coverslips were mounted in Fluoromount (Fisher Scientific Co., Pittsburgh, PA) and photographed on a Zeiss epifluorescence microscope with Kodak T-Max ASA 400 black and white film.

Immunofluorescence

Cells were plated onto glass coverslips at a density of 1–2 × 10^4 cells/cm^2. In some cases, coverslips were precoated with bovine fibronectin (Sigma Immunochemicals, St. Louis, MO) at 5 μg/ml. For routine staining of Abl, cells were fixed in absolute methanol for 5 min followed by acetone for 2 min, both at −20°C. For double immunofluorescence staining of Abl and

1. *Abbreviations used in this paper*: GAP, GTPase-activating protein; GST, gluthione S-transferase; SH3, Src homology type 3.

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F-actin, cells were fixed with glutaraldehyde/Triton X-100 according to the method of Small et al. (1978). Following fixation, cells were blocked with 5% normal donkey serum in PBS. For staining of Abl, the primary antibody was affinity-purified rabbit anti-pEX4 sera (Van Etten et al., 1989) at 1–5 μg/ml, with secondary antibody and phalloidin staining as described above.

Actin

Rabbit skeletal muscle actin was purified as described (Spudich and Watt, 1971) with gel filtration as a final step (MacLean-Fletcher and Pollard, 1980). Actin was labeled with pyrene iodoacetamide as previously described (Kouyama and Mihashi, 1981). NBD-labeled G-actin (Detmers et al., 1981) was kindly provided by Ralph Gieselmann (Brigham and Women's Hospital, Boston, MA). G-actin solutions contained 2 mM Tris, pH 7.6, 0.2 mM $CaCl_2$, 0.2 mM DTT, 0.5 mM ATP.

F-actin Pelleting Assays

Actin pelleting assays were done according to the method of Matsudaira (1992), using an Optima TLX microultracentrifuge and TL-100.1 rotor (Beckman Instruments, Palo Alto, CA). Equal amounts of starting material, supernatant, and pellet were solubilized in loading buffer, boiled, and subjected to SDS-PAGE on 5–18% gradient Laemmli gels (Matsudaira and Burgess, 1978). Gels were stained with Coomassie blue, dried between cellophane, and protein bands quantitated by densitometric scanning, utilizing a MicroTek MSF-300GS image scanner and Aldus Superpaint software (Silicon Beach). Protein concentration was determined by the Bio-Rad protein assay reagent in comparison to a standard BSA curve. Dye binding by the fusion proteins was linear in the range used. In the case of the pEX5 fusion protein (which comigrates with actin at about 42 kD), the proteins were transferred to nitrocellulose after electrophoresis, and immunoblotted with affinity-purified anti-pEX5 antibodies. The primary antibody was detected with ^{125}I-labeled protein A (kind gift of Dr. Lloyd Klickstein, Center for Blood Research, Boston, MA).

For in vitro transcription, the c-*abl* (IV) and c-*abl* (IV) ΔXB cDNAs were cloned into the pGEM-4 vector (Promega Biotec, Madison, WI), DNA templates linearized by cutting with HindIII or SalI, and RNA transcribed with SP6 RNA polymerase (Promega Biotec) to yield mRNA coding for Abl protein which is full length or lacking the COOH-terminal 165 amino acids, respectively. Rabbit reticulocyte lysates (Promega Biotec) were programmed with the appropriate *abl* RNA, Abl proteins labeled with [^{35}S]methionine (Dupont/New England Nuclear, Boston, MA), and aliquots used for cosedimentation with F-actin as described above. Following SDS-PAGE, ^{35}S-labeled Abl proteins were visualized by fluorography utilizing Amplify solution (Amersham Corp., Arlington Heights, IL).

To determine the calcium dependence of actin binding by Abl, pelleting was performed with the addition of 0.2 mM $CaCl_2$ to the assay buffer. For Scatchard analysis, r/c was plotted versus c, where r = (moles of GST-Abl bound to F-actin)/(moles of F-actin pelleted) and c = concentration of unbound GST-Abl. To investigate whether Abl and gelsolin compete for the same binding site on F-actin, GST-ΔFok and gelsolin S2-3 were simultaneously mixed with F-actin before pelleting. The molar ratio of ΔFok to actin was kept constant at 2.0, while the molar ratio of gelsolin to actin was varied over a range of 0.0 to 2.0. Equivalent amounts of supernatant and pellet fractions were analyzed by SDS-PAGE, and quantitation of fusion protein binding was performed as described above.

Fluorescence Assays of Actin Polymerization

The rate and extent of actin polymerization in the presence of various Abl proteins were determined from the increase in fluorescence of pyrene-actin that occurs when pyrene-labeled actin polymerizes, as described (Kouyama and Mihashi, 1981). Polymerization of actin was initiated by mixing 198 μl of G-actin in buffer A (2 mM Tris-HCl, pH 8.0, 0.2 mM $CaCl_2$, 0.2 mM DTT, 0.5 mM ATP) with 30 μl of solutions containing Abl protein in 10 mM Tris, pH 7.4, and then adding 12 μl of 40 mM $MgCl_2$ and 3M KCl to raise the Mg^{2+} and KCl concentrations to 2 mM and 150 mM, respectively. The total pyrine-actin concentration was 3 μM.

Native Gel Electrophoresis

Native gel electrophoresis was performed essentially as described by Safer (1989). Purified monomeric G-actin (1–3 μg) was mixed with a one- to threefold molar excess of purified GST-Abl or Abl protein in buffer G (2 mM Pipes, pH 7.0, 0.2 mM $CaCl_2$, 0.2 mM DTT, 0.2 mM ATP) and incubated on ice for 30 min. Glycerol was added to 10% and the sample loaded on a 6% polyacrylamide (30:0.8 acrylamide/bis) gel containing 25 mM Tris, 194 mM glycine, 0.5 mM $CaCl_2$, 0.2 mM DTT, and 0.2 mM ATP. Gels were prerun in the same buffer for 1 h at 4°C at 200 V, and fresh buffer added before loading samples. The proteins were detected by Coomassie blue staining. In this assay, when G-actin is mixed with a protein with G-actin binding activity, there is a shift of actin from the original position in the gel to a new position of lower electrophoretic mobility, representing formation of a complex between the two proteins (Safer, 1989).

Light Scattering

The intensity of light scattered at 90° during and after polymerization of actin was measured with a Brookhaven Instruments BI30ATN instrument and a 10 mW He-Ne laser. The reaction conditions were the same as used for fluorescence measurements.

Electron Microscopy

3 μM F-actin samples were applied to Formvar-coated copper grids, negatively stained with 1% uranyl acetate, and visualized with a JEOL 1200-EX transmission electron microscope at an accelerating voltage of 80 kV.

Results

Abl Associates with F-actin Stress Fibers In Vivo via a COOH-terminal Domain

The myristoylated form of murine c-Abl was stably expressed in NIH 3T3 fibroblasts. Double immunofluorescence staining with fluorescein-conjugated phalloidin, which specifically binds to F-actin microfilaments, demonstrates that Abl associates with F-actin stress fibers (Fig. 1 *A*). The association is punctate along the entire length of the microfilament, and there is no obvious preference for ends of filaments or adhesion plaques. In addition, Abl continues to co-localize with F-actin after treatment of cells with cytochalasin D, a drug which disrupts F-actin into small fragments and induces a major reorganization of the cytoskeleton (data not shown).

We analyzed several mutant forms of Abl for actin association. Although Src homology type 3 (SH3) domains have been identified in several actin-binding proteins, deletion of the Abl SH3 domain had no effect on actin association (data not shown). In contrast, Abl proteins which were truncated at the COOH terminus, typified by the mutant cIV K290M ΔSal (Fig. 1, *B* and *C*) lacked detectable F-actin association, implicating the 165 COOH-terminal amino acids of Abl in actin association. Truncation of the COOH-terminal 32 amino acids of Abl in the mutant cIV K290M ΔBgl also resulted in the complete lack of detectable F-actin association (Fig. 1, *B* and *C*). Abl was also truncated at the extreme COOH terminus at a unique Tth111-I site, resulting in the deletion of the three COOH-terminal amino acids (VRR). This mutant, cIV K290M ΔTth, demonstrated greatly reduced actin-association in vivo, although residual weak co-staining of actin microfilaments was detectable in all clones examined (Fig. 1, *B* and *C*). This suggests that the extreme COOH-terminal 32 amino acids of Abl are required for association of the protein with F-actin in vivo, and the COOH-terminal VRR sequence is important, though not absolutely required.

The COOH-terminal Domain of Abl Associates with F-actin after Microinjection, and Directly Binds to F-actin in a Cosedimentation Assay

The COOH-terminal 165 amino acids of Abl, denoted pEX5 for historical reasons (Konopka et al., 1984), was expressed

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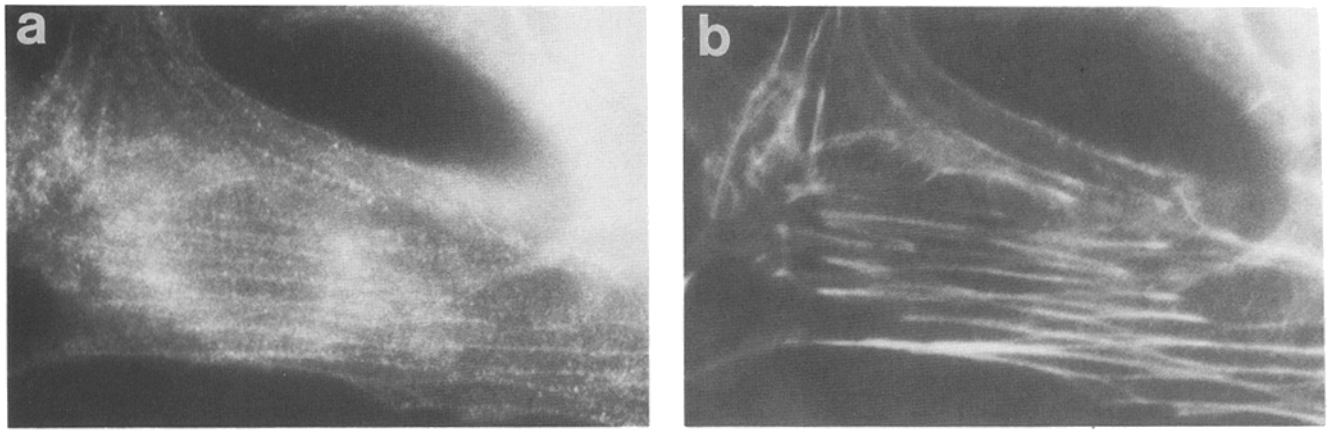

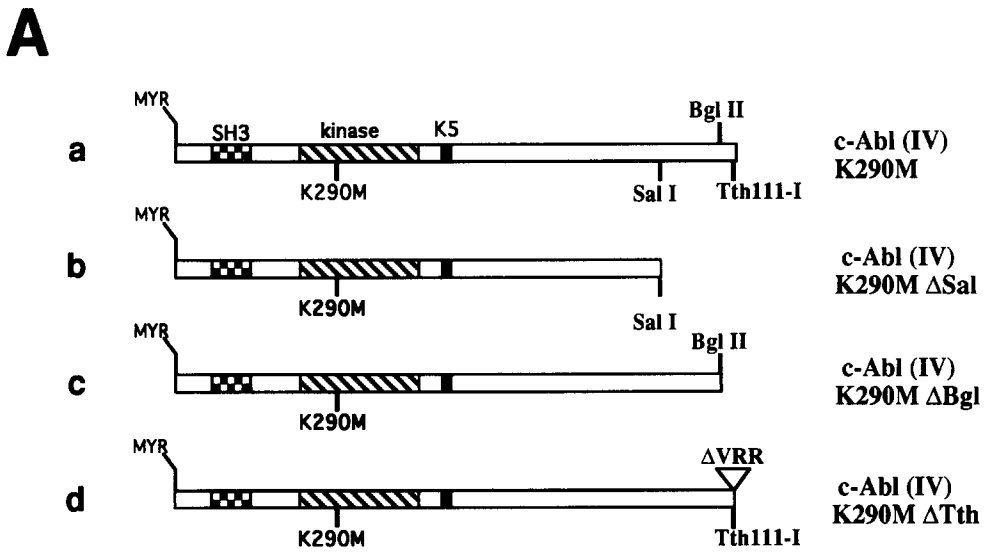

Figure 1. (*A*) Co-localization of Abl with F-actin stress filaments in NIH 3T3 fibroblasts stably expressing c-Abl (IV). Cells were fixed with glutaraldehyde and Triton X-100 (Small et al., 1978). (*a*) Primary antibody is affinity-purified rabbit α-pEX4 antibodies, secondary antibody is Texas red–conjugated donkey anti-rabbit IgG. (*b*) Same field as in *a*, visualized to detect FITC-phalloidin staining. (*B*) Schematic representation of COOH-terminal truncation mutants of c-Abl (IV). The myristoylation site, SH3 domain, kinase domain, and pentalysine nuclear localization signal are indicated. All mutants contain a kinase-inactivating point mutation in the ATP-binding site of the Abl tyrosine kinase domain (*K290M*), which improves expression without affecting actin association. (*C*) Indirect immunofluorescence of NIH 3T3 cell lines stably expressing COOH-terminal truncation mutants of c-Abl, using affinity-purified anti–pEX4 antibodies. Cells were fixed with methanol and acetone. (*a*) c-Abl (IV) K290M; (*b*) c-Abl (IV) K290M ΔSal; (*c*) c-Abl (IV) K290M ΔBgl; (*d*) c-Abl (IV) K290M ΔTth. Bar, 10 μm.

in *E. coli* as a fusion protein with GST and microinjected into NIH 3T3 fibroblasts. The GST-pEX5 fusion protein demonstrated prominent association with F-actin stress fibers one hour after injection (Fig. 2 *a*), as did a fusion protein of GST with the F-actin binding domain of gelsolin (Fig. 2 *b*). By contrast, cells injected with the GST protein alone showed only a diffuse cytoplasmic and perinuclear staining without detectable association with microfilaments (Fig. 2 *c*). Interestingly, a GST-SH3 fusion protein, containing 89 amino acids spanning the Abl SH3 domain fused to GST, failed to associate with the major F-actin stress fibers after microinjection, but did appear to associate with the very terminal portion of F-actin cytoskeleton just proximal to the lamellipodia of the cell (Fig. 2 *d*). Thus, the pEX5 domain of Abl contains determinants which can direct the association of heterologous proteins with actin microfilaments, and the Abl SH3 domain, while not capable of association with large F-actin stress fibers, does have some affinity for the terminal F-actin network.

To confirm the microinjection results, GST fusion proteins were tested for in vitro binding to F-actin in a cosedimentation assay (Matsudaira, 1992). A mixture of Abl and F-actin was pelleted by ultracentrifugation, and the amount of bound Abl detected by SDS-PAGE of supernatant and pellet fractions. A significant amount of GST-pEX5 fusion protein cosediments with F-actin (Fig. 3 *a*), where none of the paren-

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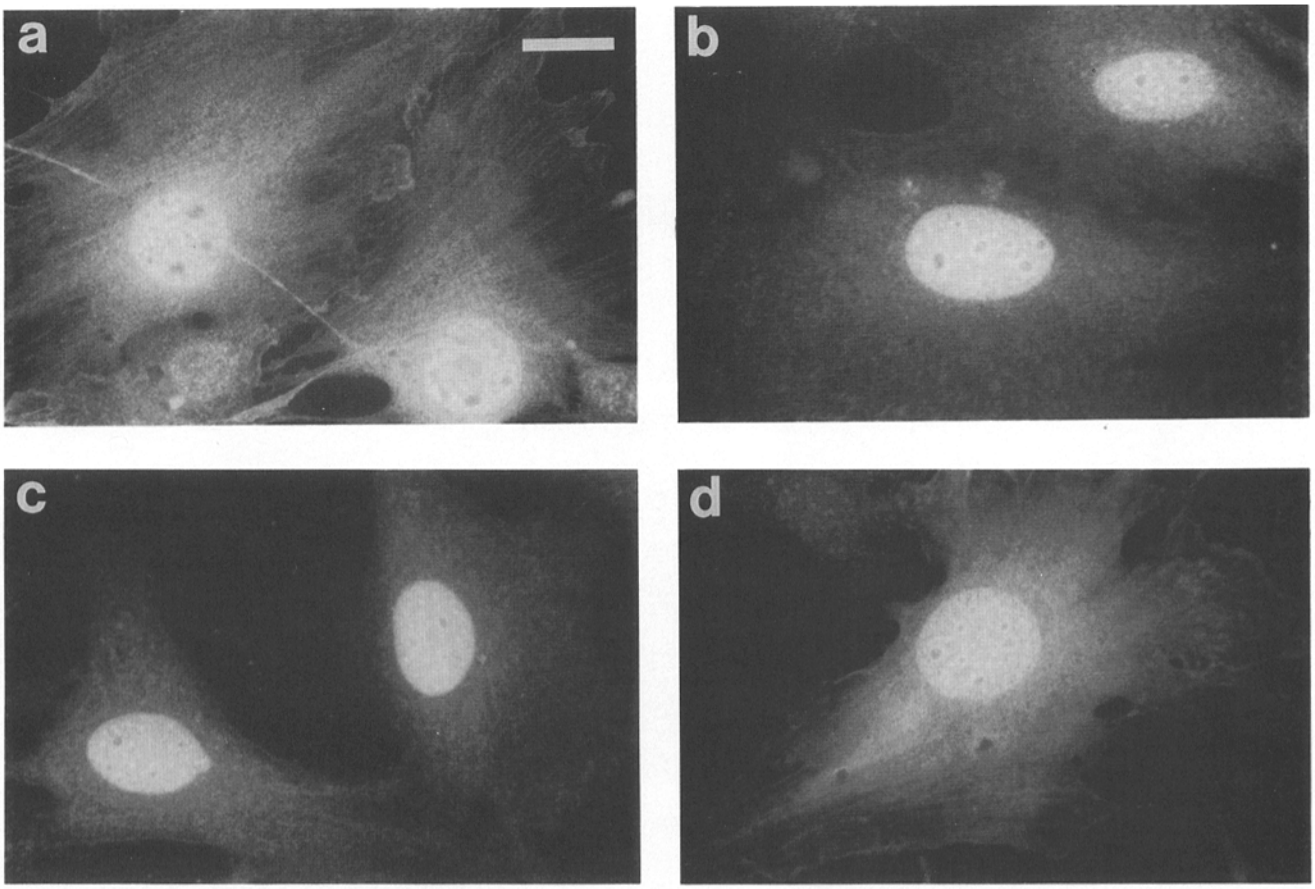

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Figure 1.

tal GST protein cosediments (Fig. 3 *b*). The GST-SH3 fusion protein exhibited no significant cosedimentation with F-actin (Fig. 3 *c*), confirming previous observations (Cicchetti et al., 1992), despite the weak association with the terminal F-actin cytoskeleton observed after microinjection. To investigate whether the native c-Abl protein also binds to F-actin, we performed actin cosedimentation assays with ^{35}S-labeled Abl proteins generated by in vitro translation. A large fraction of the full-length type IV c-Abl protein cosediments with F-actin, while Abl protein which lacks the COOH-terminal 165 amino acids shows greatly reduced but still significant actin cosedimentation (Fig. 3 *d*). Therefore, the COOH-terminal domain of Abl accounts for the majority of the Abl actin-binding activity, but other sequences in Abl appear to play a lesser but significant role. When the SH3 domain is deleted from the type IV c-Abl, the resulting protein, denoted c-Abl (IV) ΔXB (Jackson and Baltimore, 1989), still exhibits prominent cosedimentation with F-actin which is reduced to background levels when the COOH-terminal domain is also deleted (Fig. 3 *e*). Thus, in the context of the entire Abl protein, the COOH-terminal domain accounts for the majority of the actin-binding activity, with the SH3 domain contributing at a lower but significant level.

Further Mapping of the Abl F-actin Binding Domain

To better define the Abl F-actin binding site, the pEX5 domain was sequentially deleted from the NH_2- and COOH-terminal sides and the corresponding GST fusion proteins assayed for F-actin binding by microinjection and pelleting (Figs. 4 and 5). The fusion protein GST-ΔFok (containing c-Abl type IV amino acids 1015-1142) retains prominent F-actin association after microinjection (Fig. 5 *a*). The GST-ΔSca protein exhibits weaker but clearly discernible association with F-actin (Fig. 5 *b*). The fusion protein GST-ΔHinf retains some F-actin association, but also exhibits a prominent association with small linear structures at the ventral surface of the cell, suggestive of focal adhesions (Fig. 5 *c*). The identity of these structures was confirmed by double immunofluorescence with an anti-talin monoclonal antibody, and by reflection interference microscopy (data not shown). However, the fusion protein GST-ΔBgl lost all association with F-actin after microinjection (Fig. 5 *d*). Fusion proteins truncated at the Tth111-I or HinfI sites were insoluble and unable to be used in the assay, but a larger COOH-terminal truncation protein, GST-pEX5/ΔSca(C), was soluble and also showed no detectable association with F-actin after microinjection (Fig. 5 *e*). These data suggest that the Abl F-actin association domain lies in the COOH-terminal 58 amino acids.

As a complement to further mapping the actin-binding domain by microinjection, the same series of NH_2- and COOH-terminal deleted fusion proteins were assessed for their ability to cosediment with F-actin (Fig. 4). The ΔFok, ΔSca, and ΔHinf fusion proteins retained the ability to bind to F-actin, although quantitatively less of the GST-ΔSca and GST-ΔHinf

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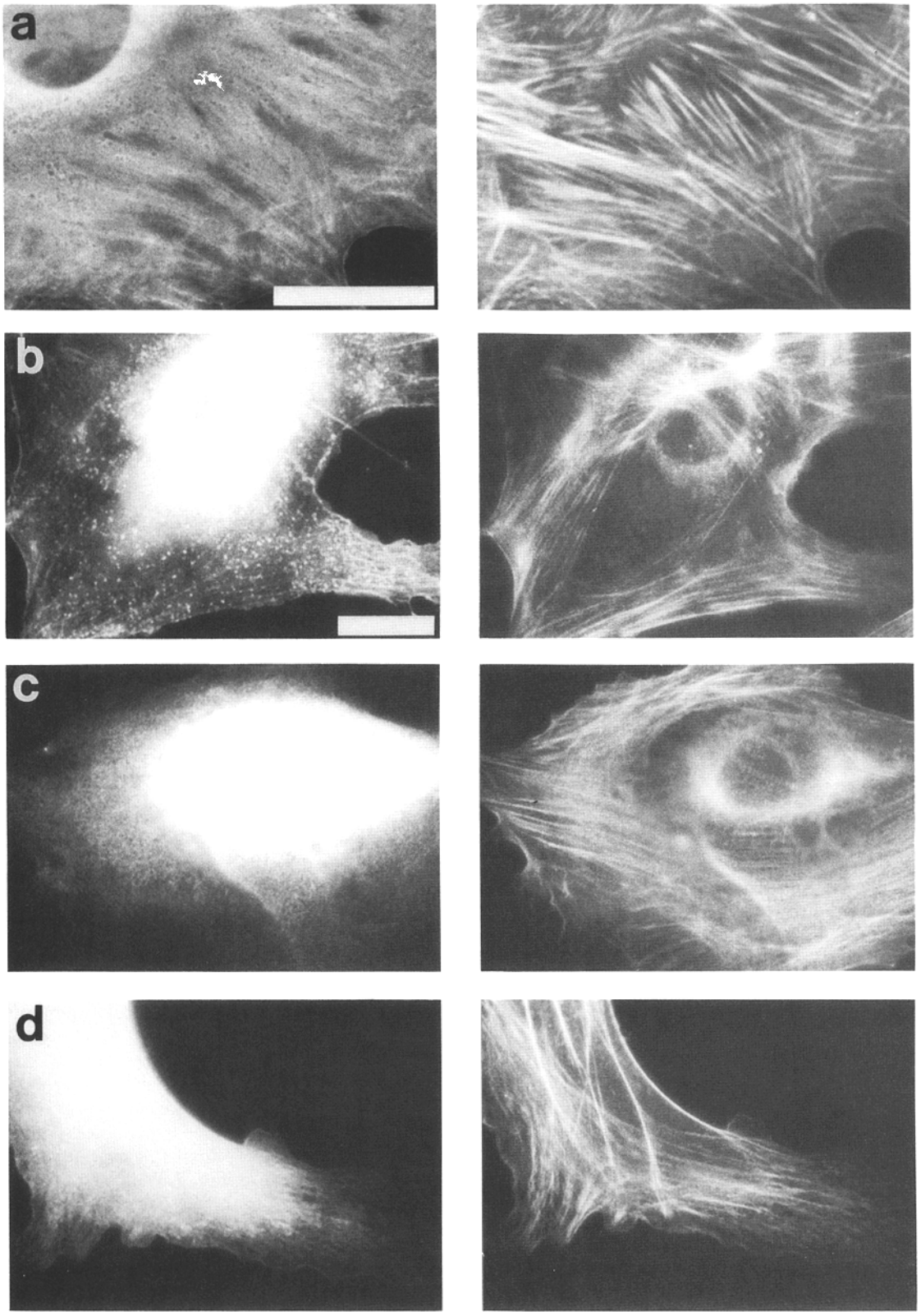

Figure 2. Microinjection of GST fusion proteins into NIH 3T3 cells. (*Left*) Primary antibody is affinity-purified rabbit anti–GST antibodies, secondary antibody is Texas red–conjugated donkey anti–rabbit IgG. (*Right*) Same field stained with FITC-phalloidin. (*a*) GST-pEX5; (*b*) GST-gelsolin S2-3. (*c*) GST. (*d*) GST-SH3. (*a* was photographed at higher magnification than *b–d*). Bars, 10 μm.

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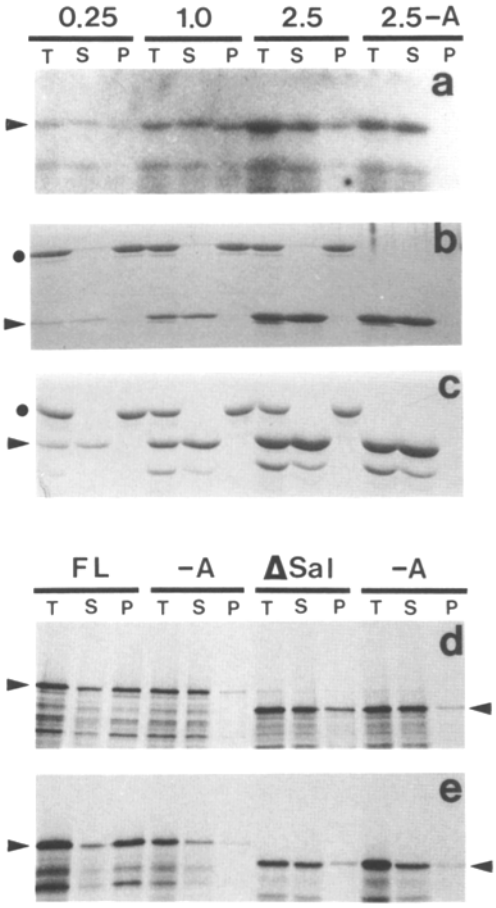

Figure 3. Actin cosedimentation assay. (*a–c*) Cosedimentation of GST-Abl fusion proteins. Each GST-Abl fusion protein was cosedimented with F-actin at 0.25, 1.0, or 2.5-fold molar excess with respect to actin, while a sample at the highest concentration (2.5-A) was centrifuged without actin. Equivalent amounts of starting material (*T*), supernatant (*S*), and pellet (*P*) were fractionated by SDS-PAGE. Dot indicates position of actin, arrowhead indicates position of GST-Abl. (*a*) GST-pEX5; (*b*) GST; (*c*) GST-SH3; (*d–e*) Cosedimentation of in vitro translated Abl proteins. Rabbit reticulocyte lysates containing ^{35}S-labeled Abl protein which was either full-length (*FL*) or lacking the COOH-terminal 165 amino acids (Δ*Sal*) were cosedimented with F-actin, while identical samples were centrifuged without actin (−*A*). Equivalent amounts of starting material (*T*), supernatant (*S*), and pellet (*P*) were fractionated by SDS-PAGE and Abl proteins visualized by fluorography. By Coomassie blue staining, more than 95% of the actin was found in the pellet fraction in each case (data not shown). (*d*) c-Abl (IV); (*e*) c-Abl (IV) with internal SH3 deletion (ΔXB). Arrowheads indicate positions of Abl proteins.

fusion proteins co-pelleted with F-actin compared with the longer fusion proteins GST-pEX5 and GST-ΔFok. By contrast, the GST-ΔBgl and the GST-ΔSca(C) fusion proteins showed no co-pelleting. Thus, Abl/F-actin association in vivo, after microinjection, and in vitro all appear to require the extreme 58 COOH-terminal amino acids of the Abl pEX5 domain. In both microinjection and pelleting assays, binding of this domain to F-actin appears to be strengthened by inclusion of NH_2-terminal pEX5 sequences present in GST-pEX5 and GST-ΔFok.

Gelsolin Competes with Abl for Binding to F-actin

Increasing amounts of the GST-ΔFok fusion protein were copelleted with F-actin and amount of Abl fusion protein in supernatant and pellet fractions quantitated by densitometry (Fig. 6 *A*). Scatchard analysis (Fig. 6 *B*) indicated a binding constant of about 18 μM for the GST-Abl/F-actin interaction, significant but somewhat weaker than the affinity of the S2-3 domain of gelsolin for F-actin (Way et al., 1992). Pelleting of GST-ΔFok with F-actin in the presence of 0.2 mM EGTA or 0.2 mM $CaCl_2$ revealed no obvious change

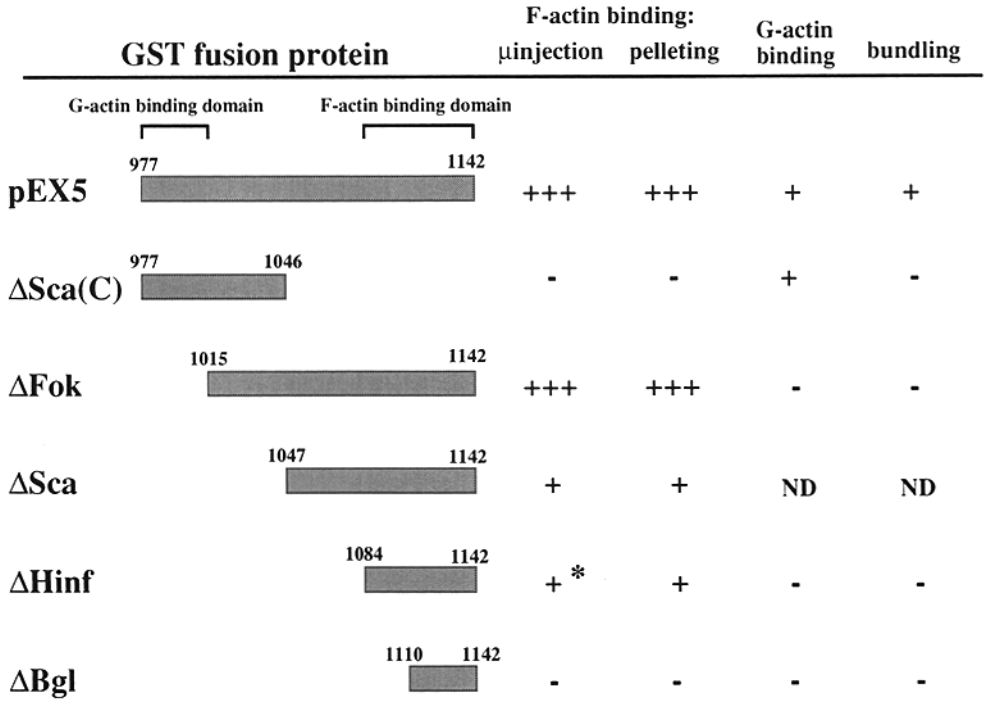

Figure 4. Schematic representation of GST-Abl fusion proteins and summary of GST-Abl actin-interaction assays. Abl sequences only are represented; all species contain an NH_2 terminal 26-kD GST moiety. Numbers correspond to amino acids from murine type IV c-Abl (Ben-Neriah et al., 1986). The asterisk indicates that the GST-ΔHinf fusion protein had an increased affinity to localize at focal adhesions after microinjection. (*ND*, not determined).

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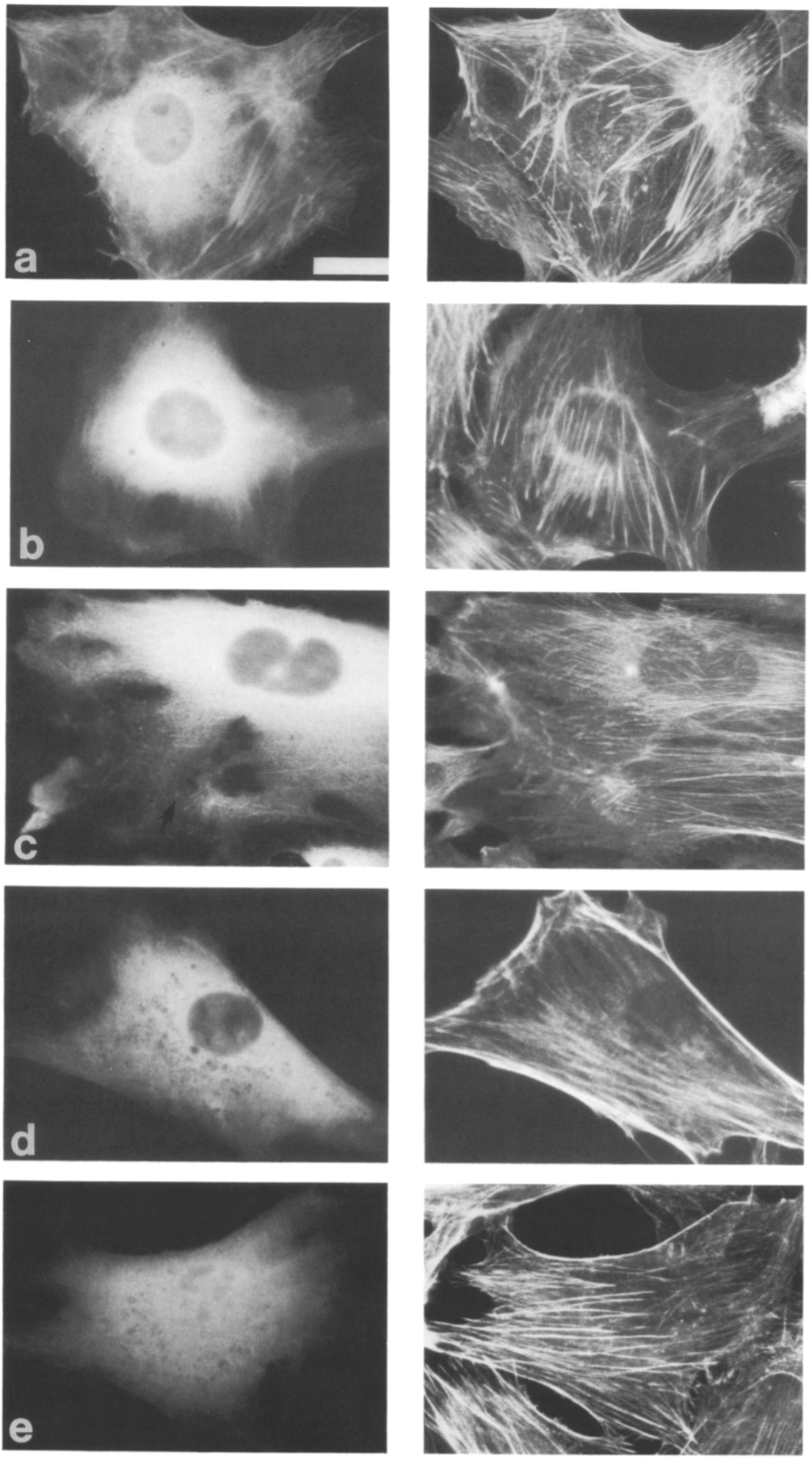

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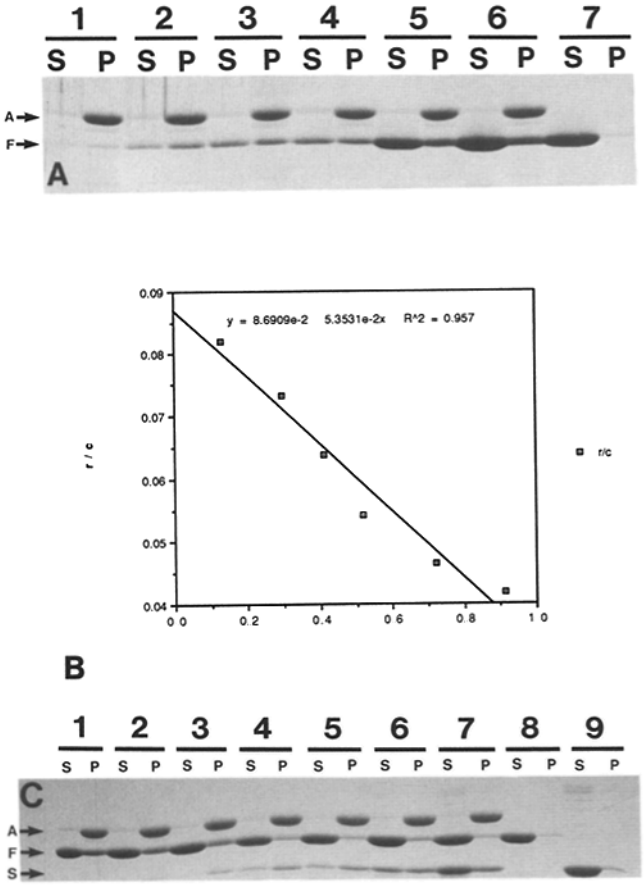

Figure 6. (*A*) Quantitation of binding of GST-ΔFok fusion protein to F-actin by cosedimentation. Equivalent amounts of supernatant (*S*) and pellet (*P*) were fractionated by SDS-PAGE. A denotes position of actin, F denotes GST-ΔFok. Lane *7*, no actin added; lanes *1–6*, increasing amounts of GST-ΔFok protein added to F-actin, from 0.2- to 3.0-fold molar excess with respect to actin. (*B*) Scatchard analysis of data derived from *A*. (*C*) Gelsolin S2-3 domain competes with GST-ΔFok for binding to F-actin. A twofold molar excess of GST-ΔFok over F-actin was used, with increasing amounts of S2-3 polypeptide. Equivalent amounts of supernatant (*S*) and pellet (*P*) were fractionated by SDS-PAGE. *A* indicates the position of actin, *F* indicates the position of GST-ΔFok, and *S* indicates the position of S2-3. Lane *1*, no S2-3 added; lanes *2–7*, increasing amounts of S2-3, from 0.2 to 2.0 molar equivalents with respect to actin; lane *8*, GST-ΔFok without actin; lane *9*, S2-3 without actin.

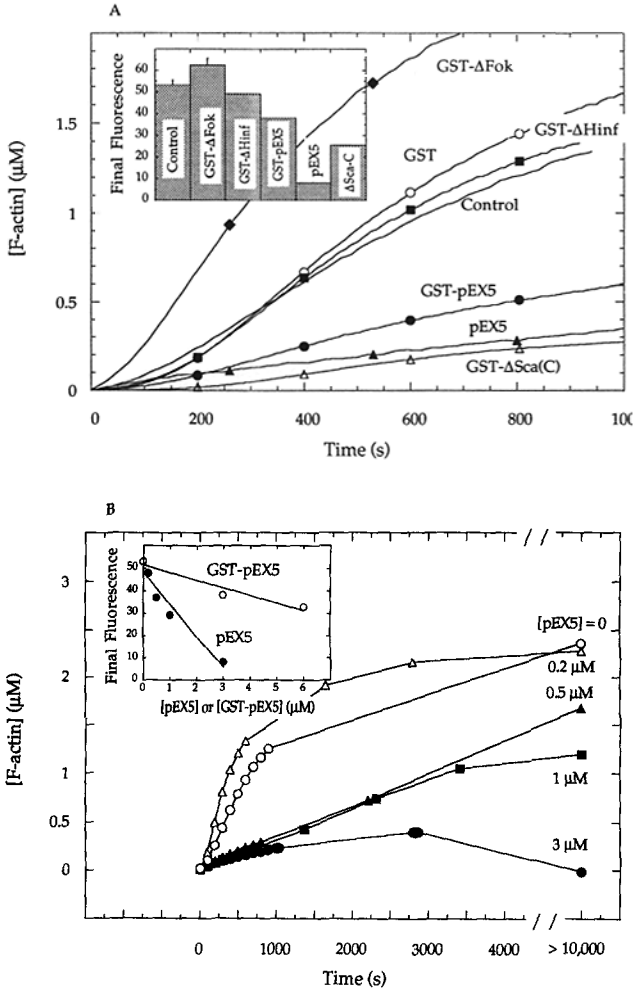

Figure 7. Inhibition of pyrene-actin polymerization by a subregion of the Abl actin-binding domain. Actin was polymerized in the presence of the indicated Abl protein, and the rate and extent of F-actin formation followed by fluorimetry. (*A*) Initial polymerization rates of 3 μM actin in the presence of 3 μM GST-Abl fusion protein or purified pEX5. (*Inset*) Final fluorescence values of the experiment in *A*, which correlates with the steady-state level of F-actin. The data for GST-ΔSca(C) was derived from separate experiments, and the values were converted to the same scale by normalization to control reactions containing actin alone. (*B*) Effect of increasing amounts of purified pEX5 polypeptide on polymerization of 3 μM pyrene-actin. (*Inset*) Plot of the final fluorescence versus the concentration of Abl for GST-pEX5 and purified pEX5.

in the affinity of Abl for F-actin (data not shown). When increasing amounts of purified gelsolin S2-3 were added to a mixture of GST-ΔFok and F-actin, the S2-3 was able to efficiently and completely compete with the GST-ΔFok for pelleting, suggesting that Abl and gelsolin bind to the F-actin microfilament via the same or overlapping binding sites (Fig. 6 *C*, lanes *2–7*). S2-3 competed efficiently with Abl for binding to F-actin even at fairly low concentrations (for example, see Fig. 6 *C*, lane *4*), confirming that it has significantly higher affinity for actin than Abl does.

The Abl pEX5 Region Contains a Distinct G-actin Binding Domain

We tested the effect of adding purified Abl fusion proteins to actin labeled with the fluorescent tag *N*-(1-pyrenyl) iodoacetamide, also known as pyrene-actin, and examined the kinetics of polymerization of the labeled actin by fluorimetry. At concentrations less than that of actin, the GST-pEX5 fusion protein showed no evidence of nucleation, capping or

Figure 5. Microinjection of NH_2- and COOH-terminal deleted GST-Abl fusion proteins into NIH 3T3 cells. Same orientation as in Fig. 2. (*a*) GST-ΔFok; (*b*) GST-ΔSca; (*c*) GST-ΔHinf; (*d*) GST-ΔBgl; arrow indicates staining of focal adhesions; (*e*) GST-ΔSca(C). Bar, 10 μm.

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severing activity (data not shown). Surprisingly, at near equimolar ratios to actin, Abl strongly suppressed the rate (Fig. 7 *A*) and extent (Fig. 7 *A*, *inset*) of pyrene-actin polymerization. We were concerned that these effects might be due to binding of Abl to newly polymerized F-actin with blocking of polymerization at the barbed end, but the GST-ΔSca(C) fusion protein, which lacks the F-actin binding domain, also inhibits pyrene-actin polymerization (Fig. 7 *A*). These effects were not observed with the parental GST protein, nor with the NH_2-terminal deleted fusion proteins GST-ΔFok and GST-ΔHinf. The increased fluorescence caused by the GST-ΔFok protein does not appear to be nucleation because the lag time before fluorescence rise is not eliminated, but may rather result from fluorescence enhancement of the pyrene probe caused by this protein binding to the side of the filament.

To show that the inhibition of pyrene-actin polymerization is not dependent on the GST sequences present in the GST-pEX5 fusion protein, we cleaved the GST moiety from the fusion protein utilizing human thrombin (Smith and Corcoran, 1992), removed free GST by binding to GSH-agarose, and used the purified pEX5 polypeptide in the pyrene-actin assay. The purified pEX5 protein alone exhibits a more potent and near stoichiometric inhibitory effect on the rate of actin polymerization (Fig. 7 *B*) and on the final fluorescence of the pyrene-actin (Fig. 7 *B*, *inset*). No effect on actin polymerization was observed with purified ΔFok polypeptide alone (data not shown). Further, the pyrene-labeled G-actin can be displaced from pEX5 and polymerized by adding an excess of unlabeled G-actin at the end of the initial polymerization reaction (data not shown). This behavior is consistent with binding and sequestration of G-actin monomers by Abl, and suggests that the NH_2-terminal 40 amino acids of the pEX5 region contains a G-actin binding domain (Fig. 4).

To directly confirm that Abl binds to G-actin, we employed a different assay for G-actin binding, native acrylamide gel electrophoresis. G-actin alone forms a single discrete band on native gels (Fig. 8 *A*, lane *5*). The GST-pEX5 fusion protein migrates as a series of diffuse bands, possibly representing oligomerization of the protein (Fig. 8 *A*, lane *3*). When a twofold molar excess of the GST-pEX5 fusion protein is mixed with G-actin, there is a significant decrease in the amount of actin migrating at the position of monomeric G-actin, without the appearance of an obvious new band of lower mobility (Fig. 8 *A*, lane *4*). No decrease is observed when the parental GST protein or the GST-ΔFok protein is mixed with G-actin (Fig. 8 *A*, lanes *6* and *8*, respectively), confirming that the NH_2-terminal 40 amino acids of the pEX5 region are responsible for this effect. In contrast, the GST-ΔSca(C) fusion protein, which contains the NH_2-terminal portion of the pEX5 region but lacks the F-actin binding domain, has a marked effect, decreasing the amount of actin migrating as monomeric G-actin by >90% (Fig. 8 *A*, lane *2*). To determine where the remainder of the G-actin is migrating, we performed the assay utilizing G-actin covalently labeled with rhodamine, and detected labeled actin after electrophoresis by observing the gel under short wave ultraviolet light (Safer, 1989). The remainder of the G-actin was found at the very top of the gel after addition of GST-pEX5 or GST-ΔSca(C) fusion protein, suggesting the formation of an Abl/G-actin complex which was either insoluble or very large (data not shown).

The presence of GST sequences is not required for this effect, because purified pEX5 polypeptide alone has a dramatic and stoichiometric effect on G-actin, eliminating essentially 100% of monomeric G-actin as the amount of pEX5 added exceeds equimolar levels (Fig. 8 *B*, lanes *1–4*). In contrast, purified ΔFok polypeptide lacking GST sequences has no effect on migration of G-actin (Fig. 8 *B*, lanes *6–9*). When rhodamine-actin is used with purified pEX5, the actin is again found at the top of the gel (data not shown). The actin is not simply degraded, because the two proteins are found to be intact after incubation if a portion of the mixture is boiled and electrophoresed on a denaturing SDS-polyacrylamide gel (data not shown). These data confirm that the pEX5 region contains a G-actin binding domain in NH_2-terminal 40 amino acids of the region. G-actin bind-

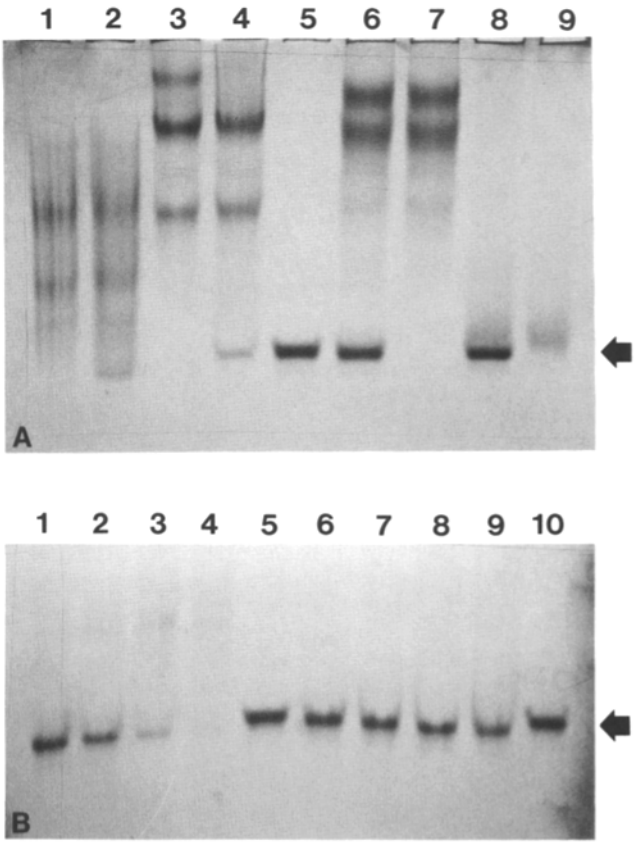

Figure 8. Native gel electrophoresis assay for G-actin binding by Abl. 3 μg actin was used in all samples, so that the amount of monomeric G-actin may be directly compared between lanes. (*A*) GST-Abl fusion proteins. Lane *1*, GST-ΔSca(C) alone; lane *2*, GST-ΔSca(C) + G-actin; lane *3*, GST-pEX5 alone; lane *4*, GST-pEX5 + G-actin; lane *5*, G-actin alone; lane *6*, GST-ΔFok + G-actin; lane *7*, GST-ΔFok alone; lane *8*, GST + G-actin; lane *9*, GST alone. (*B*) Purified pEX5 and purified ΔFok polypeptides. Lanes *1–4*, increasing amounts of pEX5, ranging from 0.5 to 2.0 molar excess with respect to actin. Lanes *6–9*, increasing amounts of ΔFok, ranging from 0.5 to 2.0 molar excess. Lanes *5* and *10*, G-actin alone. The purified Abl polypeptides migrate as diffuse species of lower mobility and are not shown; the position of monomeric G-actin is indicated by the arrows.

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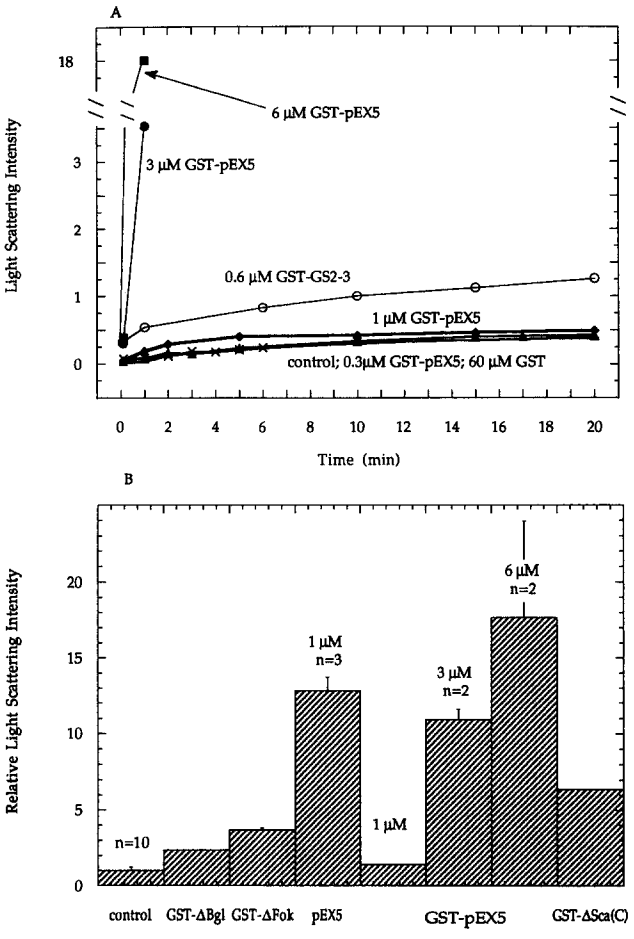

Figure 9. Effect of GST-pEX5 and other GST-Abl fusion proteins on light scattering by polymerizing actin. 3 μM unlabeled actin was polymerized in the presence of the indicated amount of GST fusion protein. (*B*) Final level of light scattering induced by various Abl proteins. GST-ΔFok, GST-ΔBgl, and GST-ΔSca(C) were present at 3 μM.

ing by Abl was not dependent on calcium because no difference was observed when $CaCl_2$ was omitted from the binding buffer and gel and 2 mM EGTA included (data not shown). However, the pEX5 domain was observed to bind significantly more efficiently to G-actin which had ADP as the bound nucleotide (Laham et al., 1993) than with ATP-actin (data not shown).

For some proteins, binding of G-actin may be detected by a change in the fluorescence emission spectrum of actin labeled with pyrene or NBD (Lee et al., 1988). There was no significant change in the fluorescence spectrum of either pyrene- or NBD-labeled actin after addition of purified pEX5 (data not shown), suggesting that Abl does not interact with Cys 374 of actin, the site of attachment of the fluorescent label.

The Abl F- and G-actin Binding Domains Mediate Bundling of F-actin

We also examined the effect of adding purified Abl fusion proteins on light scattering by polymerizing actin. The GST-pEX5 fusion protein, when present at nearly equimolar amounts relative to actin, greatly increased the rate and extent of the increase in light scattering that accompanies actin polymerization (Fig. 9, *A* and *B*). This result demonstrates that the GST-pEX5 fusion protein binds to F-actin and increases the size of the solute after actin polymerization, possibly by promoting the formation of actin filament bundles. This effect is not seen with the parental GST protein, with a GST-S2-3 fusion protein, or with actin alone, nor is it observed with any of the shorter GST–Abl fusion proteins such as GST-ΔFok and GST-ΔSca(C).

Binding of F-actin by GST fusion proteins may be complicated by the fact that GST forms dimers (Ji et al., 1992; Parker et al., 1990). Therefore, a single actin-binding site that would not link two filaments together by itself might crosslink F-actin when expressed as a dimer of GST-fusion proteins. To show that the effects of GST-pEX5 on light scattering were not related to the presence of GST in the fusion protein, we cleaved the pEX5 domain from GST sequences by thrombin, and found that purified pEX5 polypeptide alone had a near-stoichiometric effect on light scattering (Fig. 9 *B*).

To demonstrate directly that the increased light scattering was due to filament bundle formation, we examined Abl/F–actin complexes by transmission electron microscopy. GST–pEX5/F-actin preparations showed prominent crosslinking of F-actin filaments into loose bundles (Fig. 10 *A*). Similar bundling activity was detected when purified pEX5 protein was used alone (Fig. 10 *C*), but no bundles were detected with actin alone (Fig. 10 *D*), with GST (Fig. 10 *B*), or with GST-ΔFok or GST-S2-3 (data not shown). There was no obvious decoration of the filament with either pEX5 or ΔFok. The F-actin bundling activity thus appears to require both the F- and G-actin binding domains to be present. In addition, a 1:1 mixture of the GST-ΔFok and GST-ΔSca(C) fusion proteins was unable to bundle F-actin (data not shown). This confirms that two distinct domains of Abl are required for bundling, and these domains must be present in cis on the same polypeptide in order for filament bundling to occur.

We wished to determine whether the Abl G-actin sequestering activity or the F-actin binding/bundling activity would predominate under in vitro conditions. Pyrene-actin was allowed to polymerize to equilibrium, and an equimolar amount of Abl protein added. The mixture was then diluted 50-fold so that the concentration of G-actin was below the critical concentration, and the depolymerization of F-actin followed by fluorimetry. The GST-pEX5 protein significantly retarded the depolymerization of F-actin, while GST-ΔFok and GST proteins had no effect on the depolymerization rate (data not shown). This suggests that bundling by pEX5 stabilizes F-actin, and sequestration of G-actin by pEX5 does not accelerate the depolymerization. Binding of GST-ΔFok without bundling did not stabilize the F-actin. Therefore, Abl appears to promote the assembly rather than disassembly of actin under these in vitro conditions.

Discussion

We have shown that the c-Abl tyrosine kinase possesses distinct F- and G-actin binding domains in the COOH terminus of the protein, that the protein associates with the F-actin

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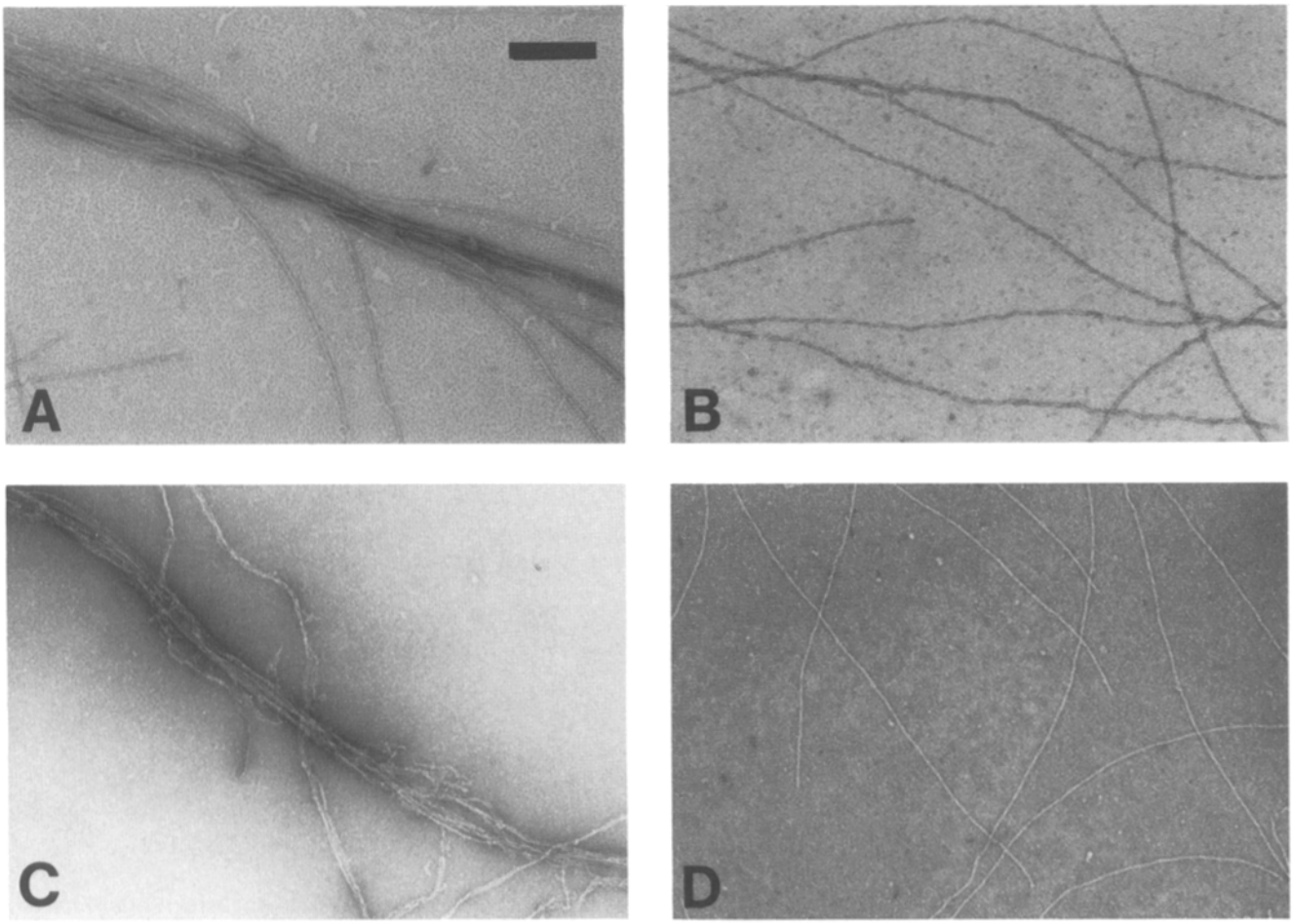

Figure 10. EM photomicrographs of Abl–actin complexes negatively stained with uranyl acetate. All samples contained 3 μM F-actin and equimolar amounts of GST or Abl proteins. (*A*) GST-pEX5; (*B*) GST alone; (*C*) purified pEX5; (*D*) actin alone.

cytoskeleton in fibroblasts, and the two actin-binding domains cooperate to bundle F-actin in vitro. These results raise the possibility that Abl could both modulate the state of the actin cytoskeleton in vitro and transduce signals from the cytoskeleton to the nucleus.

Abl F-actin Binding Domain

The Abl F-actin binding domain has been mapped to the extreme COOH terminus of the protein. The association of Abl with F-actin is observed with several different fixation conditions including methanol/acetone, paraformaldehyde, and glutaraldehyde, making it unlikely to be an artifact of fixation. All experiments were performed on NIH 3T3 cells which expressed Abl from a retroviral expression vector (Jackson and Baltimore, 1989), and overexpressed the protein 3–10-fold over the level of endogenous Abl proteins. With improved affinity-purified antisera, we have been able to detect endogenous nuclear Abl protein by immunofluorescence of NIH 3T3 cells (Van Etten, R., unpublished results), but have not yet been able to detect staining of microfilaments. Further improvements in immune reagents may allow the detection of this association in cells not overexpressing Abl.

The F-actin binding domain of murine c-Abl has also been localized to the COOH terminus by transient expression of mutant Abl proteins in Cos cells (McWhirter and Wang, 1993). In our experiments, the COOH-terminal 58 amino acids of the Abl protein are sufficient to allow binding of Abl to F-actin with a particular affinity for focal adhesions, an effect also observed in Cos cells with expression of the COOH-terminal 32 amino acids of Abl joined to sequences from the first exon of Bcr and the Abl SH3/SH2 domains. However, the GST-ΔBgl fusion protein, which contains only the 32 COOH-terminal amino acids of Abl, showed no detectable F-actin association in microinjection and pelleting assays. It is possible that inclusion of Bcr sequences in the Abl polypeptide may strengthen the Abl/F-actin interaction (McWhirter and Wang, 1991) and allow the detection of weak residual F-actin binding activity present in the COOH-terminal 32 amino acids of Abl. This might be partly due to the ability of Bcr to self-associate (Campbell et al., 1990). With regards to the COOH-terminal boundary of the F-actin binding domain, deletion of the three COOH-terminal amino acids abolished F-actin association by Abl in Cos cells (McWhirter and Wang, 1993). Expression of the cIV ΔTth mutant in NIH 3T3 fibroblasts shows that this protein has greatly reduced but detectable association with actin mi-

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crofilaments, demonstrating that the three COOH-terminal residues are not absolutely required for F-actin binding.

SH3 domains have been identified in a number of actin-binding proteins of diverse origin, including certain isoforms of myosin II (Jung et al., 1987), the cytoskeletal protein α-spectrin (Wasenius et al., 1989), and the yeast actin-binding protein ABP-1 (Drubin et al., 1990). However, in no case has an SH3 domain been directly implicated in actin binding, and actin lacks the proline-rich consensus sequence which has been identified as an SH3-binding motif (Ren et al., 1993). In Abl, the SH3 domain does not appear to account for the majority of the actin-association observed by immunofluorescence. Deletion of the entire SH3 domain yields a protein which still exhibits prominent association with F-actin, while truncation of Abl at the extreme COOH terminus abolishes detectable actin co-staining. However, the Abl SH3 domain does associate with the terminal F-actin network after microinjection, and we have observed that Abl mutants with large COOH-terminal truncations, which lack nuclear localization signals and are totally cytoplasmic, also weakly co-localize with peripheral F-actin in vivo (Van Etten, R., unpublished results). In addition, the SH3 domains of phospholipase C-γ and GRB-2 associate with F-actin microfilaments and membrane ruffles, respectively, after microinjection (Bar-Sagi et al., 1993). In the context of the full-length Abl protein, the SH3 domain contributes a minor but significant fraction of the total actin-binding activity in cosedimentation experiments, but the isolated Abl SH3 domain exhibits no significant cosedimentation with F-actin. The affinity of the isolated SH3 domain for actin may be too low to be detected by cosedimentation, but may be increased by the presence of other Abl sequences. Alternately, the Abl SH3 domain may associate with F-actin indirectly by interacting with other actin-binding proteins, some of which may be present in reticulocyte lysates. These data suggest that SH3 domains do mediate some level of association of signaling proteins with the cytoskeleton, and the combination of the SH3 domain and the COOH-terminal F-actin binding domain may modulate the distribution of Abl within the cytoskeleton.

When the Abl COOH-terminal sequence is compared with available sequences in nucleic acid and protein sequence databases, no significant similarities are identified except with other Abl family members, including the *abl*-2 or *arg* gene (Kruh et al., 1990). There are similarities between the COOH terminus of Abl and human dystrophin, and between Abl and myosin β-heavy chain, but these occur in different portions of the Abl sequence and do not involve the actin-binding domains of these proteins. There is a weak similarity between the extreme COOH terminus of Abl and a repeated domain found in several actin-binding proteins such as α-actinin, dystrophin, α-spectrin, and the homologous proteins chicken fimbrin, human L-plastin, and yeast Sac6p, all of which terminate in the motif aaXR, where a is an aliaphatic amino acid (Adams et al., 1991; McWhirter and Wang, 1993). However, Abl lacks most of the other conserved residues shared by these proteins, including a tryptophan which is absolutely conserved. Thus, the significance of this similarity is unknown. The aaXR motif at the extreme COOH terminus appears to be important, because truncation of the COOH-terminal VRR sequence from mouse c-Abl reduces but does not eliminate F-actin binding in vivo. However, this truncation may have a large effect on the conformation of the COOH-terminal domain of Abl, because it causes the corresponding GST-Abl fusion protein to become unstable and insoluble. Although the c-Abl ΔTth mutant was expressed at reasonable levels in vivo, the reduced F-actin association may be a consequence of altered conformation rather than loss of residues directly involved in F-actin binding.

It is apparent that actin-binding domains of different actin-binding proteins can be similar in their functional and binding characteristics without sharing significant homology at the amino acid level (Way et al., 1992). From competition pelleting experiments, the F-actin binding domains of gelsolin, α-actinin, and dystrophin appear to bind to the same domain on the F-actin microfilament (Way et al., 1992; Hemmings et al., 1992). Because the gelsolin S2-3 domain and the Abl F-actin binding domain also compete for binding to F-actin but neither binds G-actin, they may recognize a common determinant formed by the junction of two actin monomers in the filament, despite the lack of any conserved amino acid sequences between the two proteins. It has proven difficult to precisely define actin-binding domains through the generation and assay of point mutations (Way et al., 1992). It may be possible to better define the Abl actin-binding domain by a mutagenesis strategy using phage display technology, and by obtaining structural information from NMR or crystallographic analyses.

Abl G-actin Binding Domain and Bundling Activity

The distinct G-actin binding domain of Abl was discovered through a dramatic and specific effect of the NH_2-terminal portion of the pEX5 region on the kinetics of actin polymerization, and was confirmed by native gel electrophoresis. There is some discrepancy between the affinity of the pEX5 domain for G-actin as estimated from native gel electrophoresis, which is around 10^{-6} M, and the ability of pEX5 to inhibit pyrene-actin polymerization, which is essentially complete when equimolar amounts of pEX5 are added. In addition, there is a somewhat greater inhibition of the initial rate of pyrene–actin polymerization at lower concentrations of pEX5 than would be expected. It is possible that the native gel assay substantially underestimates the Abl/G-actin affinity, perhaps because of instability of the complexes before or during electrophoretic separation. It is also possible that bundling or capping of short F-actin filaments by pEX5 during the initial stages of polymerization might exaggerate the effect which is observed, particularly at low concentrations of Abl; for example, see (Way et al., 1992). Careful kinetic measurements using the pEX5 and ΔSca(C) polypeptides may help resolve this question.

This region of pEX5 is quite proline rich (10/39 residues), but there is no similarity to a 20-amino acid conserved motif implicated in G-actin binding by several G-binding proteins such as gelsolin, profilin, and cofilin (Vandekerckhove, 1989; Tellam et al., 1989). A phosphorylation site for protein kinase C is present adjacent to the proline-rich region (Pendergast et al., 1987), raising the possibility that a function of this domain, such as G-actin binding or filament bundling, might be modulated by phosphorylation. There is precedent for this, as the actin filament cross-linking activity of the MARCKS protein is inhibited by phosphorylation by

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protein kinase C (Hartwig et al., 1992). Abl appears to bind preferentially to the ADP form of G-actin over ATP/G-actin. In this respect, Abl resembles gelsolin and profilin, which also preferentially bind ADP/G-actin (Laham et al., 1993; Lal and Korn, 1985), as opposed to β_4-thymosin, which binds ATP/G-actin more tightly than ADP/G-actin (Carlier et al., 1993). Whether Abl influences ATPase activity or nucleotide exchange by actin monomers, as profilin and β_4-thymosin do (Goldschmidt-Clermont et al., 1992; Carlier et al., 1993), is not known. Because newly depolymerized actin is predominantly bound to ADP, while ATP/G-actin is preferentially incorporated at the barbed end of a growing actin filament, a protein such as Abl which preferentially binds ADP/G-actin might play a role in differential sequestration of G-actin pools in the cell, spatially regulating filament assembly.

Together, the Abl F- and G-actin binding domains are able to bundle F-actin filaments in vitro (Fig. 4). This effect requires that both domains be present on the same polypeptide, and does not depend on the presence of GST sequences. The F-actin binding domain alone is unable to bundle actin. Bundling of F-actin requires that an actin-binding protein have multiple actin-binding domains (Matsudaira, 1991), or else be able to self-associate and form oligomers (Lynch et al., 1987). In Abl, the combination of the two actin-binding domains appears to be sufficient for bundling, even though the G-actin binding domain alone shows no evidence of F-actin binding activity. It is possible that the combination of the two domains might result in an additional activity for actin. Gelsolin, for example, contains two G-actin binding domains and a single F-actin binding domain (Bryan, 1988). The combination of the three distinct actin-binding domains confers a new activity, F-actin severing, on the Gelsolin polypeptide, which is not present in any of the individual domains. Alternatively, actin filaments will form bundles if their negative surface charge is neutralized either by >10 mM Mg^{2+}, by lower amounts of polycations such as polylysine or arginine pentamers, or even very low amounts of basic peptides such as the 20–amino acid calmodulin-binding site of MARCKs (Hartwig et al., 1992). The pEX5 polypeptide has a modest excess of basic amino acids, but the ΔFok polypeptide has essentially the same ratio of basic to acidic residues and lacks bundling activity, suggesting that specific cross-linking is more important for the induction of actin bundles by Abl than charge neutralization.

Interaction of Abl with the Cytoskeleton In Vivo

We have shown that the F-actin binding domain of Abl is functional in vivo, but it is not known whether the G-actin binding and F-actin bundling activities are functional in the cell. Proteins with these activities can have disparate effects on the physiology of the actin cytoskeleton. G-actin binding proteins, such as β_4-thymosin, bind and sequester G-actin monomers within non-muscle cells, limiting the amount of actin available to participate in polymerization (Sanders and Wang, 1992). Actin-bundling proteins, such as fimbrin, contribute to the stability of the actin cytoskeleton and to the ultrastructure of the cell, such as the microvillus of the intestinal epithelium (Matsudaira, 1983). In the case of Abl, the G-actin binding/sequestering and F-actin binding/bundling activities would seem to be at odds with one another, with one inhibiting and the other promoting the formation of more complex actin structures. Under in vitro conditions, bundling of F-actin by Abl appeared to predominate over disassembly. Often, the actin-modulatory functions of actin-binding proteins are tightly controlled in the cell, as bundling and severing by gelsolin and villin are regulated by calcium and polyphosphoinositides (Matsudaira and Janmey, 1988). Hence, the net effect of the Abl actin-binding activities on the cytoskeleton may depend on local and spatial regulation of these activities within the cell, and on the presence and activity of other actin-modulatory proteins.

However, a simple quantitative argument suggests that Abl is unlikely to play a major role in F-actin bundling or G-actin sequestration within the cell. Quantitation of Western blot experiments has indicated that NIH 3T3 cells contain about 2.5×10^5 Abl molecules per cell (P. Jackson, unpublished results), the majority of which may be nuclear. If there are 2,500 stress fibers per cell on the average, there could be a maximum of about 100 Abl molecules per fiber, far less than the concentration of fimbrin in the microvillus (Glenny Jr. et al., 1981). In addition, the concentration of Abl in the cell would conservatively be predicted to be less than 1% of the G-actin pool. Although regional increases in the concentration of Abl, such as those found in adhesion plaques, may permit the local cytoskeletal structure to be directly influenced by Abl, it is apparent that Abl is not a major cellular actin sequestering or bundling protein.

Abl might also modulate the state of the cytoskeleton indirectly via a number of mechanisms. Although the tyrosine kinase activity of Abl is not required for F-actin binding, it is possible that actin itself or another associated protein is an important target for phosphorylation by Abl. It has been recently shown that tyrosine phosphorylation of a subset of actin in the slime mold *Dictyostelium* is associated with changes in cell morphology (Howard et al., 1993; Schweiger et al., 1992). A protein (3BP-1) has been identified which binds to the c-Abl SH3 domain and contains a region of homology to the GTPase-activating protein (GAP) for $p21^{rho}$ (Cicchetti et al., 1992), and has recently been shown to function as a GAP for Rho in vitro (Cicchetti, P., D. Baltimore, and R. Cerione, unpublished results). The association of Abl with the actin cytoskeleton may therefore serve to recruit Rho-GAP activity to the cytoskeleton via the Abl SH3 domain, perhaps regulating some of the cytoskeletal effects mediated by Rho (Ridley and Hall, 1992).

Abl may also use the dynamic cytoskeletal scaffolding to receive and transduce signals through a direct or indirect mechanism. It has recently been demonstrated that strain can be transmitted to the F-actin cytoskeleton through the interaction of integrins with their cell-surface receptors (Wang et al., 1993). It is possible that actin-associated Abl might be able to sense this mechanical signal directly and respond by tyrosine phosphorylation of a downstream effector. Alternatively, Abl may respond to signals relayed by other cytoskeletal components. Because Abl contains nuclear localization signals and a DNA binding domain, it has the potential to transfer signals from the cell surface and cytoskeleton directly to the nucleus. Overexpression studies have shown the protein to be present at some level at plasma membrane, cytoskeleton, cytoplasm, and nucleus in all cells, and there is as yet no evidence for movement of Abl between various compartments, save for the observation that all transforming Abl proteins are wholly cytoplasmic (Van

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Etten et al., 1989). Elucidation of the intracellular trafficking pattern of Abl will be essential to understanding signal transduction by this complex protein.

The effect of actin binding on transformation by activated forms of Abl is not clear-cut. The actin-binding domain appears not to be required for fibroblast transformation by activated forms of c-Abl, because this domain can be deleted from $p160^{v\text{-}abl}$ or from the SH3-deleted c-Abl mutant ΔXB without an appreciable effect on transformation efficiency (Prywes et al., 1985; Jackson and Baltimore, 1989; Jackson, P., unpublished observations). However, McWhirter and Wang found a small effect of deleting the actin-binding domain on transformation of Rat-1/v-*myc* cells or factor-dependent B-lymphoid cells by the $P210^{bcr/abl}$ protein (McWhirter and Wang, 1993). Bcr/Abl proteins are weaker transforming proteins than v-Abl, and it is possible that a modest dependence of transformation on actin-association may be detected with these proteins when none is observed with activated c-Abl.

The large 90-kD COOH-terminal region of c-Abl is unique to members of the Abl family and is not present in other members of the Src family. It is now apparent that a major role of the Abl COOH terminus is to confer several novel localizing functions upon the protein, including association with the actin cytoskeleton, signals for nuclear localization, and a domain mediating binding to DNA (Kipreos and Wang, 1992). The Abl COOH terminus appears to be essential for proper function of the protein during central nervous system development in *Drosophila* (Gertler et al., 1989; Henkemeyer et al., 1990) and lymphoid development in mice (Schwartzberg et al., 1991; Tybulewicz et al., 1991). Although the precise role of these COOH-terminal functions in the physiology of Abl is not understood, the present studies offer the potential to directly examine the contribution of the actin-binding domains in these and other genetic systems.

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