ABSTRACT

Oligomerization of the nonreceptor tyrosine kinase c-Abl can activate its transforming potential. Domains mediating oligomerization within the BCR-ABL and TEL-ABL oncoproteins are required for transforming activity, and fusion of inducible dimerization domains to c-Abl can generate chimeric proteins with dimerization-dependent transforming activity. We have found that c-Abl oligomerizes at high levels of expression in COS cells. This interaction is dependent on kinase activity and an intact NH2-terminal region of c-Abl. A binding partner of c-Abl, Abelson-interactor-1 (Abi-1), similarly oligomerizes in COS cells. An oligomeric form of Abi-1 interacts with Abl both in vitro and in mammalian cells. These results suggest the possibility that oligomerization of Abl kinases, perhaps involving regulation by their interaction partners, may play a role in modulation of kinase activity in both normal and oncogenic processes.

INTRODUCTION

The proto-oncogene c-abl encodes a nonreceptor tyrosine kinase that is ubiquitously expressed and tightly regulated in vivo. The mechanisms leading to activation of the c-Abl kinase are only partially understood. A variety of stimuli including several genotoxic agents and growth factors up-regulate its activity. For example, activation of c-Abl by IR2 requires the kinase encoded by ATM, the gene mutated in ataxia telangiectasia (1, 2). IR appears to activate c-Abl through serine phosphorylation of the kinase domain by ATM. In contrast to the IR-induced pathway, growth factor-stimulated activation of a membrane-associated pool of c-Abl is associated with tyrosine phosphorylation of the kinase domain by the Src family kinases (3). Thus, phosphorylation of either specific serine or tyrosine residues within the c-Abl kinase domain appears to favor an active confirmation.

Several studies have indicated that domains of c-Abl outside the catalytic region participate in regulation of its enzymatic function. In particular, mutations within the SH3 domain of c-Abl can induce its kinase activity and reveal its transforming potential (4, 5). Both intramolecular and intermolecular mechanisms have been proposed for the inhibitory function of the SH3 domain. Mutational analysis has provided evidence for intramolecular inhibitory contacts of the SH3 domain with the catalytic domain and with the linker region between the SH2 and catalytic domains (6). Recently, the NH2-terminal 80 residues of c-Abl have been implicated in autoinhibition (7). Several proteins that bind to the c-Abl SH3 domain have been identified, but among these interaction partners only the antioxidant protein Pag/MSP23 has been shown to inhibit c-Abl kinase activity in mammalian cells (8).

In contrast to c-Abl, oncogenic Abl variants arising from chromosomal translocations are constitutively active and tyrosine-phosphorylated. The translocation events that generate BCR-ABL and TEL-ABL oncoproteins fuse regions of BCR and TEL containing oligomerization domains to c-Abl sequences. These oligomerization domains are required for transforming activity. In BCR-ABL, amino acids 1–63 of BCR comprise a coiled-coil tetramerization domain (9). Mutation or deletion of this region in BCR-ABL abolishes its ability to transform Rat-1/myc fibroblasts and to abrogate interleukin-3 dependence in Ba/F3 cells. The importance of oligomerization is additionally suggested by the functional replacement of this region with the leucine zipper of the yeast transcription factor GCN4 (10). In TEL-ABL, the TEL region includes a helix-loop-helix domain that mediates oligomerization (11). Deletions within this region abolish the ability of TEL-ABL to transform Rat-1 fibroblasts, Ba/F3 cells, and primary murine bone marrow cells. In addition, oligomerization-defective mutants of BCR-ABL and TEL-ABL all display low or undetectable levels of phosphotyrosine, suggesting that oligomerization is required for kinase activity and autophosphorylation.

Studies of chimeric proteins joining c-Abl to inducible dimerization domains have provided further evidence for the importance of oligomerization in activating oncogenic Abl kinases. Fusion of the extracellular and transmembrane domains of the erythropoietin receptor to the NH2-terminus of c-Abl creates a chimeric receptor/kinase that displays erythropoietin-dependent transforming activity both in vitro and in vivo (12). Addition of the hormone-binding domain of the estrogen receptor to the COOH terminus of a nontransforming truncated c-Abl generates a fusion protein with hormone-dependent transforming activity in vitro (13). Both chimeric proteins display ligand-dependent tyrosine phosphorylation. Recently, introduction of the FKBP (FK506-binding protein) module into c-Abl has been shown to mediate chemically induced dimerization resulting in kinase activation and cellular transformation in both fibroblastic and hematopoietic cells (14). Taken together, these results strongly indicate that dimerization alone can promote autophosphorylation and activation of c-Abl.

We have investigated whether wild-type c-Abl has the potential to oligomerize on its own. Using a combination of epitope-tagged and untagged proteins, we demonstrate oligomerization of overexpressed c-Abl in COS cells. This interaction is kinase-dependent and requires the NH2-terminal region of c-Abl. We also show that Abi-1 has the potential to oligomerize. In addition, an oligomeric form of Abi-1 can bind to Abl in vitro. These results suggest the possibility that oligomerization of c-Abl, perhaps involving its interaction partners, may contribute to regulation of its kinase activity.

MATERIALS AND METHODS

Cell Culture and Transfections. COS cells and 293T cells were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mm l-glutamine, and antibiotics. COS cells were transfected with 10 μg of DNA per 2 × 106 cells as described previously (15). NIH-3T3 cells were grown on coverslips in DMEM supplemented with 10% calf serum and were transfected using LipofectAMINE Plus (Life Technologies, Inc.) according to the manufacturer’s recommendations.

Immunofluorescence. Cells were fixed for 20 min in ice-cold methanol and blocked in 1% goat serum (Sigma) for 1 h. Myc-tagged Abi-1 was detected using anti-myc antibody (Santa Cruz; 1:200) for 1 h followed by FITC-conjugated goat antimouse immunoglobulin (Sigma; 1:200) for 1 h. Antibody incubations included 1% goat serum. Cells were washed three times with PBS.
after each antibody incubation. Cells were visualized by confocal microscopy (Zeiss).

Mammalian Expression Constructs. Myc-tagged Abi-1, HA-tagged Abi-1, and kinase-deficient v-Abl expression vectors were described previously (15). Myc-tagged and untagged c-Abl expression constructs were generated by inserting PCR-amplified fragments of murine type IV c-abl into pMT21.

Antibodies. Polyclonal (K-12) and monoclonal (8E9) antibodies against Abi-1 were purchased from Santa Cruz Biotechnology and Pharmingen, respectively. Anti-HA and anti-myc antibodies were obtained from BABCO and Santa Cruz Biotechnology, respectively. FITC-conjugated antiserum against immunoglobulin antibody was purchased from Sigma.

Fusion Proteins and In Vitro Binding Assays. The GST-Abi construct was created by inserting sequences encoding amino acids 622–978 of the c-Abl into the bacterial expression vector pGEX-2TK (Pharmacia). The GST-Abi-1 construct was described previously (15). GST fusion proteins were immobilized on glutathione Sepharose 4B (Pharmacia). In vitro binding assays were otherwise performed as described previously (15).

Immunoprecipitation and Western Blot Analysis. Cells for Abi-1 coimmunoprecipitation experiments were washed in PBS (PBS plus 0.4 mM Na3VO4 and 0.4 mM EDTA) and lysed in TNENI [50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 10 mM EDTA, 0.5% NP40, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 μg/ml pepstatin] plus 10 mM NaF and 1 mM Na3VO4. Cells for v-Abl coimmunoprecipitation studies were washed in PBS and lysed in EBC buffer [50 mM Tris-HCl (pH 7.5), 120 mM NaCl, 1 mM EDTA, 0.5% NP40, and 0.5 mM DTT] plus 10 mM NaF, 1 mM Na3VO4, and protease inhibitors. At 24 h after transfection, cells for c-Abl coimmunoprecipitation experiments were washed in PBS and lysed in radioimmunoprecipitation assay buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS] plus 10 mM NaF and protease inhibitors. Preparation of cell lysates, immunoprecipitation, and Western blot analysis were performed as described previously (15).

RESULTS

Oligomerization of c-Abl in COS Cells. To detect intermolecular interactions between c-Abl proteins, we tested the ability of an untagged c-Abl protein to coimmunoprecipitate with an epitope-tagged c-Abl protein. We coexpressed in COS cells an untagged COOH-terminal deletion mutant (amino acids 1–978) and a myc-tagged full-length murine type IV c-abl protein. Western blot analysis with anti-Abl antibody of total cell lysates confirmed roughly equivalent protein expression (Fig. 1, bottom). Myc-tagged and untagged c-Abl proteins were clearly distinguishable by their difference in mobility in SDS-PAGE. Myc-tagged full-length c-Abl was immunoprecipitated with anti-myc antibody, and the precipitated proteins were resolved by SDS-PAGE and immunoblotted with anti-Abl antibody to detect both the myc-tagged full-length c-Abl and coprecipitated untagged Abl (1–978). We found that the two c-Abl proteins strongly associate in COS cells (Fig. 1, top). The nearly equal levels of the two proteins in the immunoprecipitate suggest that very high portions of the proteins are in the complex. Anti-myc antibody did not precipitate untagged c-Abl in the absence of myc-tagged c-Abl. A kinase-inactivating mutation on untagged c-Abl protein reduced the amount of untagged c-Abl that coprecipitated with myc-tagged c-Abl. Disruption of kinase activity on both c-Abl proteins abolished the interaction and virtually eliminated detectable phosphotyrosine on the myc-tagged c-Abl (Fig. 1, top, Lane 8 and middle, compare Lanes 6 and 8). Overall, these results suggest that c-Abl can oligomerize in a kinase-dependent manner and autophosphorylate by a trans mechanism.

Oligomerization of c-Abl Requires Its NH2 Terminus. We next mapped the regions on Abi required for this interaction. The deletion mutants examined in these studies are depicted in Fig. 2A. We tested the ability of myc-tagged full-length c-Abl and a myc-tagged c-Abl deletion mutant lacking the SH3 and SH2 domains to bind to a series of untagged deletion mutants of c-Abl. Both myc-tagged c-Abl proteins bound to all three of the untagged COOH-terminal deletion mutants (Fig. 2B). The shortest of the three COOH-terminal deletion mutants is truncated immediately following the kinase domain. We also found that the COOH-terminal region alone was insufficient for oligomerization (Fig. 2C). These findings indicate that deletion of the NH2-terminal 250 residues from one binding partner does not disrupt oligomerization, but additional truncation removing the first 509 amino acids appears to abolish the interaction. The results from experiments presented in Fig. 2, B and C, are summarized in Fig. 2A.

Subcellular Localization of Abi-1. To investigate whether Abi-1 is likely to associate with the nuclear or cytoplasmic pool of c-Abl, we determined the subcellular localization of Abi-1. NIH-3T3 cells grown on glass coverslips were transiently transfected with myc-tagged Abi-1. Fixed cells were stained with anti-myc antibody and FITC-conjugated antiserum against immunoglobulin, and the stained cells were visualized by confocal microscopy. Cells overexpressing myc-tagged Abi-1 revealed a distinct filamentous pattern throughout the cytoplasm (Fig. 3), suggestive of a cytoskeletal association. Some of the transfected cells exhibited an additional punctate cytoplasmic staining (Fig. 3, bottom). No staining was detected in cells transfected with empty vector or untagged Abi-1, or in Abi-1-transfected cells incubated with secondary antibody alone (data not shown). We conclude that Abi-1 has the potential to interact with the cytoplasmic pool of c-Abl, the subset likely to be involved in regulation of signal transduction from a number of mitogenic receptors.

Oligomerization of Abi-1 in COS Cells. We also tested whether Abi-1 can interact with itself. Several combinations of HA-tagged and myc-tagged Abi-1 proteins were coexpressed in COS cells. Cell lysates were prepared, proteins were immunoprecipitated with anti-HA antibody, and the precipitated proteins were resolved by SDS-PAGE and immunoblotted with anti-myc antibody to detect coprecipitated myc-tagged proteins. The membranes were reprobed with anti-HA antibody to confirm equal loading of HA-tagged proteins. Total cell lysates were subjected to Western blot analysis with anti-HA and anti-myc antibodies to determine levels of protein expression. As shown in Fig. 4A, myc-tagged Abi-1 coimmunoprecipitated with HA-tagged Abi-1. In similar experiments, HA-tagged Abi-1 also coimmunoprecipitated with myc-tagged Abi-1 (data not shown). Treatment of serum-starved cells with EGF for 5 min did not affect this association. Deletion of the SH3 domain from both constructs also failed to disrupt the interaction, indicating that the SH3 domain is not required. In contrast, deletion of amino acids 1–85 from HA-tagged Abi-1 abolished the association. Inclusion of only amino acids 86–97 did not affect this interaction.
acids 1–210 in myc-tagged Abi-1 was sufficient for binding to HA-
Abi-1 (Fig. 4B). These results suggest that the NH₂ terminus of Abi-1
is important for oligomerization. This region of Abi-1 was shown
previously to be necessary for association with the Sos proteins,
suggesting possible interplay between homomeric and heteromeric
interactions of Abi-1 (15).

**Binding of Oligomeric Abi-1 to Abl in Vitro.** We next tested
whether oligomeric Abi-1 could bind to c-Abl in vitro. For these
studies we used a GST-Abl fusion protein that includes amino acids
622–978 of type IV c-Abl. This fragment of c-Abl includes the
proline-rich motif that binds to the Abi-1 SH3 domain but does not
include the Abl SH3 or kinase domain. Therefore, Abi-1 deletion
mutants that lack the SH3 domain are unable to interact directly with
GST-Abl(622–978). GST and GST-Abl(622–978) were immobilized
on glutathione Sepharose and incubated with cell lysates prepared
from COS cells expressing myc-tagged Abi-1 proteins. Bound pro-
teins were recovered and immunoblotted with anti-myc antibody
to detect precipitated Abi-1 proteins. A fraction of the glutathione
Sepharose eluate was resolved by SDS-PAGE and stained with Coo-
massie brilliant blue to confirm the presence of roughly equivalent
amounts of GST and GST-Abl(622–978). As shown in Fig. 5A,
myc-tagged full-length Abi-1 bound to GST-Abl(622–978). In con-
trast, myc-tagged Abi(1–210) did not associate with GST-Abl(622–
978). However, in the presence of full-length Abi-1, Abi(1–210) did
bind to GST-Abl(622–978). As expected, GST alone did not associate
with either myc-tagged Abi-1 protein. These results demonstrate that
full-length Abi-1 can mediate bridging of an NH₂-terminal Abi-1
fragment to GST-Abl(622–978) in a ternary complex. This finding
suggests that Abi-1 can interact in an oligomeric form with Abl
proteins.

**Binding of Oligomeric Abi-1 to v-Abl in Vivo.** To confirm that
the binding of Abi-1 to Abl observed in vitro can occur in mammalian
cells, we tested whether full-length Abi-1 could also mediate the
binding of the Abi-1 NH₂-terminal fragment to v-Abl in COS cells.
Similar to GST-Abl(622–978), v-Abl lacks the Abl SH3 domain and is unable to bind directly to Abi(1–210). COS cells were cotransfected with a kinase-deficient v-Abl expression vector and plasmids encoding myc-tagged full-length Abi-1, Abi(1–210), or both full-length and truncated Abi-1. Abl proteins were immunoprecipitated with anti-Abl antibodies, and precipitated proteins were immunoblotted with anti-myc antibody to detect any coprecipitated Abi-1 proteins. As shown in Fig. 5B, full-length Abi-1 coimmunoprecipitated with v-Abl. In contrast, Abi(1–210) was not detected in v-Abl immunoprecipitates. However, in the presence of full-length Abi-1m Abi(1–210) coimmunoprecipitated with v-Abl. Although the signal is low, the specific band was repeatedly detected above the background bands seen in control lanes. It should be noted that in the absence of transfected v-Abl a small amount of full-length Abi-1 is still detected in anti-Abl immunoprecipitates, presumably because of the presence of endogenous c-Abl. These results suggest that Abi-1 can interact in an oligomeric form with v-Abl in mammalian cells.

**DISCUSSION**

Activation of receptor tyrosine kinases through dimerization and autophosphorylation induced by either ligand binding or oncogenic mutation is well-documented (16). A similar role for oligomerization in both normal and oncogenic activation of nonreceptor tyrosine kinases has yet to be demonstrated. Experiments with both naturally occurring oncoproteins and genetically engineered fusion proteins indicate that oligomerization of the Abi tyrosine kinase is sufficient to activate its transforming potential. However, little is known about whether oligomerization regulates the nontransforming functions of c-Abl. Our results suggest that oligomerization of overexpressed c-Abl does occur by a kinase-dependent mechanism in COS cells. Although our experiments use highly overexpressed levels of protein and therefore may not directly recapitulate events at physiological concentrations, it is quite possible that stimuli known to activate the kinase activity of c-Abl may also induce locally elevated concentrations of c-Abl and promote increased oligomerization. For example, integrin stimulation triggers activation of c-Abl and its transient recruitment to focal adhesions, but it remains unclear whether activation occurs before or after relocalization (17). We believe that our preliminary findings should encourage additional investigation into the possible role of oligomerization of c-Abl and its interaction partners in regulation of its kinase activity.

Localization of c-Abl to the nucleus and cytoplasm involves recognition of both nuclear import and export signals (18, 19). Oligomerization may also regulate the subcellular localization of c-Abl. It has been reported that chemically induced dimerization of c-Abl leads to exclusively cytoplasmic localization in Ba/F3 cells (14). Our results indicate that efficient oligomerization of overexpressed c-Abl requires kinase activity, suggesting the possibility that dimerization-dependent cytoplasmic localization may also be kinase-dependent. However, integrin regulation of nuclear-cytoplasmic transport of kinase-defective c-Abl expressed in c-Abl-deficient fibroblasts is reported similar to that of endogenous c-Abl in wild-type cells (17). If oligomerization of endogenous c-Abl can be demonstrated, it will be
interesting to see if it occurs by strictly kinase-dependent or independent mechanisms and if it is required for nuclear-cytoplasmic transport.

Several binding partners of c-Abl have been identified recently, but for the most part the functional significance of these interactions remains unclear. We have found that one of these partners, Abi-1, oligomerizes in COS cells. Our demonstration that overexpressed Abi-1 can oligomerize and interact with c-Abl in an oligomeric form suggests that some of these binding partners may be capable of bridging or blocking Abl-Abl interactions. The filamentous and punctate staining of myc-tagged Abi-1 in NIH-3T3 cells is consistent with the findings of others testing the localization of Abi-1 and Abi-2 in cultured neurons, and suggests that proteins that bind Abi-1 may also be concentrated in these structures (20). Abi-1 and Abi-2 have been shown recently to localize to sites of actin polymerization at the tips of lamellipodia and filopodia (21). Taken together with the capacity of Abi-1 to bind actin and the defect in platelet-derived growth factor-induced membrane ruffling seen in c-Abl-deficient cells, these results suggest that some of these binding partners may be capable of bridging or blocking Abl-Abl interactions. The filamentous and punctate staining of myc-tagged Abi-1 in NIH-3T3 cells is consistent with the findings of others testing the localization of Abi-1 and Abi-2 in cultured neurons, and suggests that proteins that bind Abi-1 may also be concentrated in these structures (20). Abi-1 and Abi-2 have been shown recently to localize to sites of actin polymerization at the tips of lamellipodia and filopodia (21). Taken together with the capacity of c-Abl to bind actin and the defect in platelet-derived growth factor-induced membrane ruffling seen in c-Abl-deficient cells, these results suggest the possibility that homomeric and heteromeric interactions among c-Abl and its interaction partners may play an important role in cytoskeletal reorganization and cell motility.

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Homo- and Hetero-Oligomerization of the c-Abl Kinase and Abelson-Interactor-1

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