THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 15, Issue of April 14, pp. 10779–10787, 2000

Printed in U.S.A.

Regulation of the Rapamycin and FKBP-Target 1/Mammalian Target of Rapamycin and Cap-dependent Initiation of Translation by the c-Abl Protein-tyrosine Kinase*

(Received for publication, July 1, 1999, and in revised form, January 18, 2000)

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The c-Abl protein-tyrosine kinase is activated by ionizing radiation and certain other DNA-damaging agents. The rapamycin and FKBP-target 1 (RAFT1), also known as FKBP12-rapamycin-associated protein (FRAP, mTOR), regulates the p70S6 kinase ($p70^{S6k}$) and the eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1). The present results demonstrate that c-Abl binds directly to RAFT1 and phosphorylates RAFT1 *in vitro* and *in vivo*. c-Abl inhibits autophosphorylation of RAFT1 and RAFT1-mediated phosphorylation $p70^{S6k}$. The functional significance of the c-Abl-RAFT1 interaction is further supported by the finding that eIF4E-dependent translation in mouse embryo fibroblasts from $Abl^{-/-}$ mice is significantly higher than that compared in wild-type cells. The results also demonstrate that exposure of cells to ionizing radiation is associated with c-Abl-mediated binding of 4E-BP1 to eIF4E and inhibition of translation. These findings with the c-Abl tyrosine kinase represent the first demonstration of a negative physiologic regulator of RAFT1-mediated 5′ cap-dependent translation.

The cellular response to anti-cancer drugs that damage DNA includes cell cycle arrest, activation of DNA repair, and in the event of irreparable DNA damage, the induction of apoptosis. However, the signals that determine cell fate, which is survival or apoptosis, are largely unknown. The c-Abl non-receptor tyrosine kinase is activated in cells exposed to ionizing radiation (IR)[1] and diverse types of DNA damage (1–3). Recent studies have shown that activation of c-Abl by genotoxic stress is associated with interaction of c-Abl and the p53 tumor suppressor in the G_1 arrest response (4). Other downstream signals of c-Abl include induction of the stress-activated protein kinase and p38 mitogen-activated protein kinase by genotoxic agents (1, 3, 5).

Whereas c-Abl contains a DNA binding domain (6), direct interaction with damaged DNA could contribute to the induction of c-Abl activity. The interaction of c-Abl with sensors of DNA damage, such as the DNA-dependent protein kinase (DNA-PK), has also lent support to a model in which c-Abl is a target for activation by an upstream effector (2, 7). Other studies have demonstrated that c-Abl associates with the ataxia-telangiectasia mutated (ATM) protein and that ATM may also contribute to activation of c-Abl in the response to DNA damage (8, 9). Although the precise mechanisms responsible for the activation of c-Abl remain unclear, the potential consequence of the interactions between c-Abl and DNA-PK or ATM may contribute to cell fate in response to DNA damage. Importantly, cells defective in DNA-PK or ATM are hypersensitive to the lethal effects of ionizing radiation (IR) (10–12), whereas c-Abl-deficient cells are resistant to IR-induced killing (13–14). c-Abl-deficient cells are also resistant to killing by certain other anti-cancer drugs that damage DNA, such as 1-β-D-arabinofuranosyl cytosine (13). Thus, whereas DNA-PK and ATM may be directly responsible for activation of c-Abl, DNA strand breaks appear to represent an initial signal. Accumulation of DNA double strand breaks also occurs during recombination. In this context, c-Abl associates with the Rad51 recombinational repair protein (14) and may be essential in meiotic recombination (15).

The ubiquitously expressed rapamycin and FKBP-target 1 (RAFT1), also known as FRAP, mTOR, and RAPT1, contains a protein kinase domain that is related to those of the DNA-PK, ATM, and MEC1 checkpoint gene products (16–23). Recent studies have shown that RAFT1 directly phosphorylates $p70^{S6k}$ and the eukaryotic initiation factor 4E (eIF4E)-binding protein (4E-BP1; also known as PHAS-I) (24–26). 4E-BP1 and the related 4E-BP2 protein control the activity of eIF4E (27–29). eIF4E is present in rate-limiting amounts in most cells and plays a central role in cap-dependent initiation of protein translation (30). 4E-BP1 and 4E-BP2 bind to eIF4E by a mechanism dependent on their phosphorylation state (31–33). In contrast to hyperphosphorylated 4E-BP1 and 4E-BP2, the hypophos-

* This work was supported by United States Public Health Service Grant CA75216 (to S. K.) awarded by the NCI, National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: IR, ionizing radiation; mTOR, mammalian target of rapamycin; FRAP, FKBP12 rapamycin-associated protein; RAFT1, rapamycin and FKBP12 target 1; eIF4E, eukaryotic initiation factor-4E; 4E-BP1, 4E-binding protein 1; $p70^{S6k}$, p70 S6 kinase; PI 3-K, phosphatidylinositol 3-kinase; PIK, phosphatidylinositol kinase; DNA-PK, DNA-dependent protein kinase; ATM, ataxia-telengectasia mutated; 4E-BP2, 4E-binding protein 2; anti-P-Tyr, anti-phosphotyrosine; GST, glutathione *S*-transferase; LUC, luciferase; PAGE, polyacrylamide gel electrophoresis; Gy, gray; HA, hemagglutinin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

phorylated forms interact with eIF4E (32, 33). Thus, stimulation of cells with growth factors or serum results in the phosphorylation of 4E-BP1/4E-BP2 and thereby their dissociation from eIF4E to relieve translational inhibition.

Since DNA-PK and ATM both interact with the c-Abl tyrosine kinase, and RAFT1 is a member of this family, we asked whether c-Abl contributes to the regulation of RAFT1-mediated cap-dependent translation. The results demonstrate that c-Abl regulates RAFT1 and thereby binding of 4E-BP1 to eIF4E. The functional significance of the c-Abl/RAFT1 interaction is supported by the finding that c-Abl inhibits eIF4E-dependent translation.

MATERIALS AND METHODS

Cell Culture—Human U-937 myeloid leukemia cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Fibroblasts from 4E-BP1$^{-/-}$, 4E-BP1$^{+/+}$, Abl$^{-/-}$, and wild-type mice and NIH3T3, 293T, MCF-7, and MCF-7/c-Abl (K-R) cells were grown in DMEM containing 10% fetal bovine serum and antibiotics. Irradiation was performed at room temperature using a Gammacell-1000 (Atomic Energy of Canada, Ottawa, Ontario, Canada) under aerobic conditions with a ^{137}Cs source emitting at a fixed dose rate of 0.76 Gy/min as determined by dosimetry. Cell viability was assessed by trypan blue exclusion.

Transient Transfections—Cells were transiently transfected with HA-RAFT1 with or without c-Abl by the calcium phosphate method as described (3). After 24–36 h, the cells were harvested, lysed, and subjected to immunoprecipitation with anti-HA (Roche Molecular Biochemicals) or anti-c-Abl (Santa Cruz Biotechnology, Santa Cruz, CA). MCF-7 and MCF-7/c-Abl (K-R) cells were transiently transfected with HA-RAFT1 by LipofectAMINE (Life Technologies, Inc.). After transfection, cells were irradiated at 20 Gy and harvested at the indicated times. Total cell lysates were subjected to immunoprecipitation with anti-HA and analyzed by immunoblotting with anti-P-Tyr or anti-HA antibodies. The blots were scanned, and signal intensities were determined by densitometric analysis (UltroScan; Amersham Pharmacia Biotech).

Immunoprecipitation and Immunoblot Analysis—Cell lysates were subjected to immunoprecipitation with anti-HA, anti-RAFT1 (C-17, Santa Cruz Biotechnology, Santa Cruz, CA), or anti-c-Abl (Santa Cruz Biotechnology) as described (3). After washing the precipitates three times with lysis buffer, proteins were separated by SDS-PAGE and transferred to nitrocellulose filters. The filters were then analyzed by immunoblotting with anti-c-Abl (AB-3, Oncogene Science), anti-HA, anti-P-Tyr (4G10, Upstate Biotechnology Inc., Lake Placid, NY), or anti-RAFT1 (mouse monoclonal; provided by Drs. Joseph Avruch or Robert Abraham). The antigen-antibody complexes were visualized by chemiluminescence (ECL, Amersham Pharmacia Biotech). In certain experiments, anti-c-Abl was preincubated with the peptide used as immunogen, and the mixture of antibody and peptide was incubated with cell lysate for 2 h. Anti-c-Abl immunoprecipitates were analyzed by immunoblotting with anti-RAFT1 antibody. Signal intensities were determined by densitometric analysis (UltroScan; Amersham Pharmacia Biotech).

Fusion Protein Binding Assays—Purified GST, GST-c-Abl, or GST-Abl SH3 were incubated with lysate from cells overexpressing HA-RAFT1 for 1 h at 4 °C as described (34). The adsorbates were analyzed by immunoblotting with anti-HA.

In Vitro c-Abl Kinase Assays—Cells were transiently transfected with a kinase-dead mutant of RAFT1 (HA-RAFT1 D2357E). Lysates were subjected to immunoprecipitation with anti-HA, and the beads were incubated with recombinant purified active or heat-inactivated c-Abl in the presence of [γ-^{32}P]ATP in kinase buffer at 30 °C for 15 min. Reactions were terminated by the addition of SDS sample buffer and boiling. The proteins were separated by SDS-PAGE and analyzed by autoradiography.

In Vitro RAFT1 Kinase Assays—Cells were transiently transfected with 8 μg of HA-RAFT1 cDNA. After 24 h, cells were washed and lysed in buffer A (50 mM HEPES-KOH, pH 7.4, 40 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 1.5 mM sodium vanadate, 50 mM NaF, 10 mM sodium pyrophosphate, 10 mM sodium β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 6 mg/ml chymostatin, and 1 mg/ml of leupeptin, aprotinin, pepstatin, and antipain). Lysates were subjected to immunoprecipitation with anti-HA and incubated with kinase active or inactive c-Abl. Kinase reactions were performed as described above. Reaction products were washed twice with buffer B (50 mM HEPES-KOH, pH 7.4, 40 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 0.05% SDS), twice with buffer B containing 0.5% Triton X-100 and 0.5 M Li, and once with 50 mM HEPES-KOH, pH 7.4, 150 mM NaCl and 1 mM dithiothreitol. RAFT1 kinase assays were performed in a 30-μl reaction mixture containing 200 ng of GST-p70^{S6k} Y-A mutant fusion protein and [γ-^{32}P]ATP at 30 °C for 20 min. Reactions were stopped by the addition of SDS sample buffer. After boiling for 5 min, proteins were separated in 4–12% SDS-polyacrylamide gradient gels and analyzed by autoradiography.

4E-BP1 Phosphorylation Assays—293T cells were transfected with HA-4E-BP1 and increasing amounts of pSRα c-Abl. After 24–36 h, the cells were harvested, lysed, and subjected to immunoblot analyses by anti-HA antibody (Roche Molecular Biochemicals).

Luciferase Activity Assays—Cells were transiently cotransfected with empty vector or c-Abl and pGEMLUC/pol/CAT (27) (2 μg) using Lipofectin as described (35). NIH3T3 cells were transfected with pcDNA3-LUC-pol-CAT, and after 5 h, serum was added with or without 50 ng/ml rapamycin. Cell extracts were prepared 24 h post-transfection and assayed for luciferase activity using a Enhanced Luciferase Assay Kit (Analytical Luminescence Laboratories, Ann Arbor, MI).

Isolation of RNA and RNase Protection Assays—293T cells were transiently transfected with pSRα or pSRα c-Abl. After 48 h, total RNA was isolated using the single step guanidinium isothiocyanate method as described (36). Internally labeled RNA probes were made by *in vitro* transcription with T3 or T7 RNA polymerase in the presence of [α-^{32}P]UTP as described (36). DNA templates were removed by RQ1 DNase digestion followed by phenol/chloroform extraction. RNA was hybridized overnight to ^{32}P-labeled antisense RNA probes specific for GAPDH (PharMingen, San Diego, CA) and luciferase (37). The hybridization products were digested with a T1/T2 mixture at 37 °C for 1.5 h, and the resulting samples were resolved in 6% denaturing polyacrylamide gels. Routinely, 20 μg of RNA samples were used for RNase protection. The protected bands were quantitated using a Packard Instant Imager.

7-Methyl-GTP and eIF4E Affinity Chromatography—Cells were exposed to 20 Gy IR and harvested at various time intervals. To purify endogenous eIF4E, 30 μl of a 50% slurry of 7-methyl-GTP-Sepharose (Amersham Pharmacia Biotech) were added to the cleared cell lysates and incubated for 45 min at 4 °C. After washing the resin twice with buffer D (50 mM HEPES, pH 7.4, 40 mM NaCl, 2 mM EDTA, 0.1% Triton X-100), bound proteins were eluted with 2× SDS sample buffer, resolved by SDS-PAGE, transferred to nitrocellulose filters, and analyzed by immunoblotting with anti-4E-BP1 (clone 11208 (27)). m^7-bound proteins were also analyzed by immunoblotting with anti-eIF4E or anti-eIF4G (provided by Dr. Sonenberg, Montreal, Canada).

Metabolic Labeling—Fibroblasts from 4E-BP1$^{-/-}$ and 4E-BP1$^{+/+}$ mice and U-937 cells were serum-starved for 48 h and preincubated for 1 h in methionine-free medium. [^{35}S]Methionine (100 μCi) and 10% fetal bovine serum were then added to the cultures. Total cell lysates prepared in buffer I (150 mM NaCl, 50 mM Tris, pH 7.5, 0.5% Nonidet P-40) were analyzed for incorporated radioactivity in trichloroacetic acid precipitates.

RESULTS

Association of c-Abl with RAFT1—To determine whether c-Abl associates with RAFT1, total cell lysates from MCF-7 cells were subjected to immunoprecipitation with anti-c-Abl, and the resulting precipitates were analyzed by immunoblotting with anti-RAFT1. The results demonstrate binding of c-Abl and RAFT1 that is blocked by preincubation of the anti-c-Abl antibody with a peptide used as the immunogen (Fig. 1*A*). Similar results were obtained in a reciprocal experiment in which anti-RAFT1 immunoprecipitates were analyzed by immunoblotting with anti-c-Abl (Fig. 1*B*). Because IR activates c-Abl (1–4, 38), we investigated whether IR affects the interaction between c-Abl and RAFT1. The results demonstrate that exposure of cells to IR had little if any effect on the association of these proteins (Fig. 1*B*). The finding that RAFT1 and c-Abl also associate constitutively in 293T cells indicates that the interaction between these proteins is not specific to MCF-7 cells (data not shown). To assess the stoichiometry of the interaction between c-Abl and RAFT1, MCF-7 cells were subjected to immunoprecipitation with anti-c-Abl, and the lysates

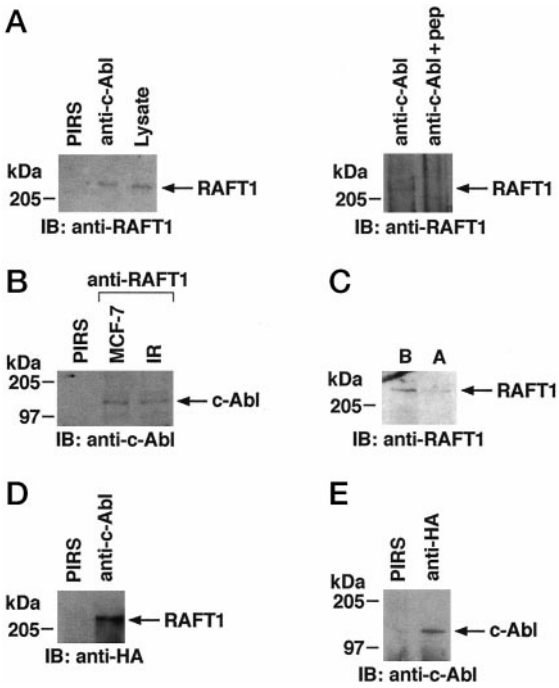

FIG. 1. **c-Abl associates with RAFT1.** *A, left panel,* total cell lysates from MCF-7 cells were subjected to immunoprecipitation with anti-c-Abl or preimmune rabbit serum (*PIRS*). The protein precipitates and total cell lysate were separated by SDS-PAGE and transferred to nitrocellulose filters. The filters were analyzed by immunoblotting (*IB*) with anti-RAFT1 antibody. *Right panel,* total cell lysates from MCF-7 cells were subjected to incubation with anti-c-Abl antibody or with a mixture of anti-c-Abl + peptide (used as immunogen). Precipitated proteins were analyzed by immunoblotting with anti-RAFT1. *B,* MCF-7 cells were exposed to 20 Gy IR and harvested at 3 h. Total cell lysates were subjected to immunoprecipitation with anti-RAFT1 or preimmune rabbit serum. The protein precipitates were separated by SDS-PAGE and transferred to nitrocellulose. The filters were analyzed by immunoblotting with anti-c-Abl antibody. *C,* MCF-7 cell lysates were subjected to immunoprecipitation with anti-c-Abl antibody. Lysates before (*B*) and after (*A*) immunoprecipitation were analyzed by immunoblotting with anti-RAFT1. *D,* 293T cells were transiently transfected with HA-RAFT1 and c-Abl. Cell lysates were subjected to immunoprecipitation with anti-c-Abl and analyzed by immunoblotting with anti-HA antibody. *E,* 293T cells were transiently transfected with HA-RAFT1 and c-Abl. Cell lysates were subjected to immunoprecipitation with anti-HA, and the precipitates were analyzed by immunoblotting with anti-c-Abl.

before and after immunoprecipitation were analyzed by immunoblotting with anti-RAFT1. The filters were scanned to determine the percentage of c-Abl associated with RAFT1. The results demonstrate that approximately 50% of RAFT1 is present in the complex with c-Abl (Fig. 1*C*). Similar results were obtained when lysates from 293T cells before and after immunoprecipitation with anti-c-Abl were analyzed by immunoblotting with anti-RAFT1 (data not shown). To confirm the interaction between RAFT1 and c-Abl, we transiently overexpressed HA-RAFT1 with c-Abl in 293T cells and analyzed anti-c-Abl immunoprecipitates by immunoblotting with anti-HA. Reactivity of anti-HA with a 289-kDa protein supported the coprecipitation of RAFT1 with c-Abl (Fig. 1*D*). In the reciprocal experiment, anti-HA immunoprecipitates were subjected to immunoblot analysis with anti-c-Abl. The results confirmed the identification of a complex containing RAFT1 and c-Abl (Fig. 1*E*).

To determine whether RAFT1 is phosphorylated on tyrosine in cells, MCF-7 and MCF-7/c-Abl (K-R) cells overexpressing a kinase-dead c-Abl in which lysine 290 has been mutated to arginine (K290R (39)) were transiently transfected with HA-RAFT1 and exposed to IR, and anti-HA immunoprecipitates were analyzed by immunoblotting with anti-P-Tyr. The results demonstrate that, by contrast to MCF-7/c-Abl (K-R) cells, irradiation of MCF-7 cells is associated with a nearly 3-fold increase in tyrosine phosphorylation of RAFT1 (Fig. 2, *A* and *B*). These findings provided support for IR-induced tyrosine phosphorylation of RAFT1 by a c-Abl-dependent mechanism.

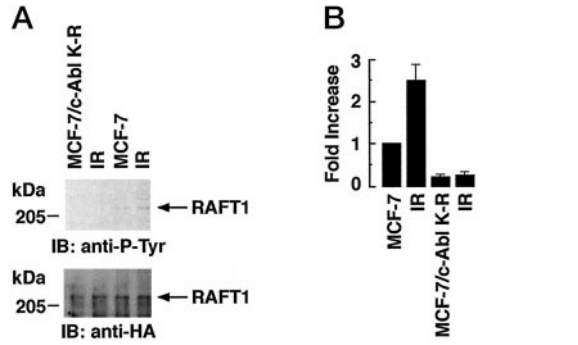

FIG. 2. **Tyrosine phosphorylation of RAFT1 in the response to ionizing radiation.** *A,* MCF-7 and MCF-7/c-Abl (K-R) cells were transiently transfected with HA-RAFT1, exposed to IR at 20 Gy, and harvested after 1 h. Total cell lysates were subjected to immunoprecipitation with anti-HA, and the precipitates were analyzed by immunoblotting (*IB*) with anti-P-Tyr (*upper panel*) or anti-HA (*lower panel*). *B,* signal intensities from the anti-P-Tyr immunoblotting experiments described in *A* were determined by densitometric analysis, and the results are expressed as the mean ± S.D. of three independent experiments.

Direct Binding of c-Abl to the C-terminal Domain of RAFT1—Binding of RAFT1 and c-Abl was further studied by incubating cell lysates with glutathione *S*-transferase (GST) fusion proteins. Adsorbates obtained with GST-c-Abl, and not with GST, demonstrated binding to RAFT1 (Fig. 3*A*). Adsorbates obtained with GST-Abl SH3 also demonstrated binding to RAFT1 (Fig. 3*A*). The c-Abl SH3 domain binds to proline-rich sequences with the consensus P*XXXX*P*XX*P (40, 41). The identification of such a sequence for c-Abl SH3 binding in RAFT1 (**P**GTYD**P**NO**P**; amino acids 2141–2149) supported a potential direct interaction between these two proteins. To determine whether the interaction between c-Abl and RAFT1 is direct, anti-HA immunoprecipitates from HA-RAFT1-transfected cells were subjected to SDS-polyacrylamide gel electrophoresis and then transferred to a nitrocellulose filter. Analysis of the filter by incubation with purified GST-Abl-SH3 and immunoblotting with anti-GST showed binding of c-Abl to RAFT1 (Fig. 3*B*). These findings demonstrate that c-Abl directly associates with RAFT1.

To define the region of RAFT1 responsible for the association with c-Abl, Myc-tagged N-terminal or C-terminal RAFT1 were transiently overexpressed in 293T cells. Total cell lysates were then subjected to immunoprecipitation with anti-Myc and analyzed by immunoblotting with anti-c-Abl. As a positive control, anti-c-Abl immunoprecipitates were also analyzed by immunoblotting with anti-c-Abl. The results demonstrate that c-Abl associates with the C-terminal (containing the potential c-Abl-SH3 domain binding sequence, amino acids 2141–2149), and not the N-terminal, fragment of RAFT1 (Fig. 3*C* and data not shown). Similar results were obtained in a reciprocal experiment in which anti-c-Abl immunoprecipitates were analyzed by immunoblotting with anti-Myc (Fig. 3*D* and data not shown).

Phosphorylation of RAFT1 by c-Abl—To assess in part the functional significance of the interaction between c-Abl and RAFT1, we investigated whether RAFT1 is a substrate for c-Abl. 293T cells were transfected with a vector expressing a kinase-inactive HA-RAFT1 D2357E mutant (25). Cell lysates were subjected to immunoprecipitation with anti-HA, and the precipitates were incubated with purified kinase-active c-Abl, heat-inactivated c-Abl, or buffer in the presence of [γ-^{32}P]ATP.

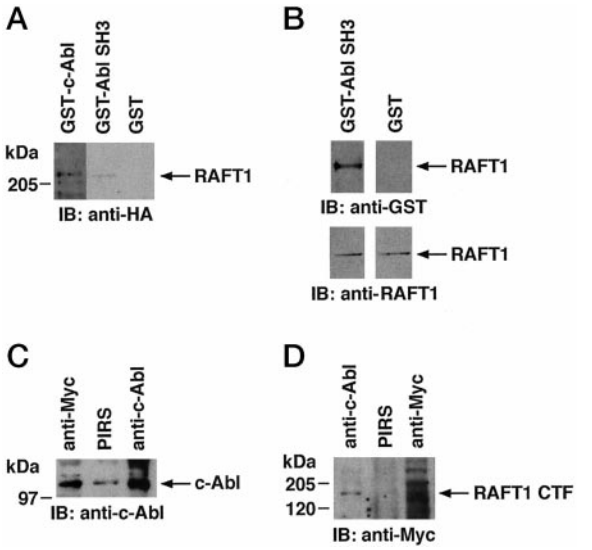

FIG. 3. **Direct interaction of c-Abl and RAFT1.** *A,* 293T cells were transiently transfected with HA-RAFT1. Cell lysates were incubated with GST, GST-c-Abl, or GST-Abl-SH3 domain fusion proteins. The protein adsorbates were analyzed by immunoblotting with anti-HA. *B,* 293T cells were transiently transfected with HA-RAFT1. Total cell lysates were subjected to immunoprecipitation with anti-HA. The immunopurified RAFT1 protein was separated by SDS-PAGE. Two identical filters were then incubated with GST or GST-c-Abl SH3 fusion proteins for 1 h at room temperature. The filters were then analyzed by immunoblotting (*IB*) with anti-GST antibody (*upper panels*) and with anti-RAFT1 antibody (*lower panels*). *C,* 293T cells were transiently transfected with Myc-RAFT1 C-terminal fragment (*CTF*). Total cell lysates were subjected to immunoprecipitation with anti-Myc, anti-c-Abl, or preimmune rabbit serum (*PIRS*). The protein precipitates were analyzed by immunoblotting with anti-c-Abl antibody. *D,* 293T cells were transiently transfected with Myc-RAFT1 CTF. Total cell lysates were subjected to immunoprecipitation with anti-c-Abl, anti-Myc, or preimmune rabbit serum, and the protein precipitates were analyzed by immunoblotting with anti-Myc antibody.

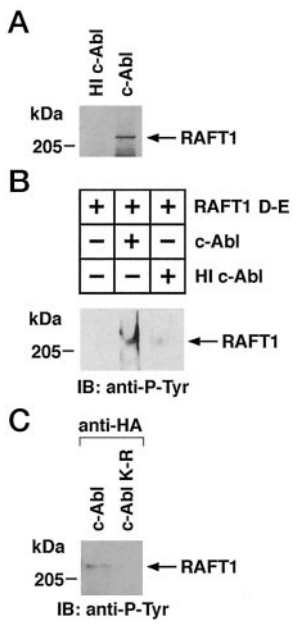

FIG. 4. **Phosphorylation of RAFT1 by c-Abl.** *A,* 293T cells were transiently transfected with the HA-RAFT1 D2357E mutant. Cell lysates were subjected to immunoprecipitation with anti-HA. Protein adsorbates were incubated with recombinant purified kinase active or inactive (HI) c-Abl in the presence of [γ-^{32}P]ATP for 15 min at 30 °C. Reactions were terminated by the addition of SDS sample buffer. Proteins were resolved by SDS-PAGE and analyzed by autoradiography. *B,* 293T cells were transiently transfected with HA-RAFT1 D2357E, and total cell lysates were subjected to immunoprecipitation with anti-HA. The immunoprecipitates were then incubated with buffer, purified recombinant c-Abl protein, or heat-inactivated c-Abl protein in the presence of cold ATP for 15 min at 30 °C. Phosphorylated proteins were resolved by SDS-PAGE, transferred to nitrocellulose filters, and analyzed by immunoblotting with anti-P-Tyr. *C,* 293T cells were transiently cotransfected with HA-RAFT1 D2357E and wild-type c-Abl or c-Abl (K-R). Total cell lysates were subjected to immunoprecipitation with anti-HA, and the precipitates were analyzed by immunoblotting with anti-P-Tyr.

Analysis of the reaction products by autoradiography demonstrated that RAFT1 is a substrate of c-Abl *in vitro* (Fig. 4*A*). To confirm tyrosine phosphorylation of RAFT1 *in vitro*, kinase reactions were also performed in the presence of cold ATP, and the products were analyzed by immunoblotting with anti-P-Tyr. The results demonstrate c-Abl-mediated tyrosine phosphorylation of RAFT1 (Fig. 4*B*). To assess phosphorylation of RAFT1 *in vivo*, 293T cells were transiently cotransfected with HA-RAFT1 D2357E and kinase-active c-Abl or the dominant negative c-Abl (K-R) (42). Lysates were subjected to immunoprecipitation with anti-HA, and the precipitates were analyzed by immunoblotting with anti-P-Tyr. The results demonstrate that coexpression with kinase-active c-Abl, and not c-Abl (K-R), results in tyrosine phosphorylation of HA-RAFT1 D2357E on tyrosine (Fig. 4*C*). Taken together, these results support c-Abl-mediated phosphorylation of RAFT1.

Inactivation of RAFT1 by c-Abl—The functional significance of the c-Abl-RAFT1 interaction was further determined by assessing RAFT1 activity in the presence of c-Abl. Anti-HA immunoprecipitates from 293T cells transiently transfected with HA-RAFT1 were incubated with or without purified recombinant c-Abl in the presence of cold ATP. As a control, 293T cells were transiently transfected with the RAFT1 D2357E mutant. Following incubation with c-Abl for 30 min, the complexes were washed under stringent conditions to remove ATP and c-Abl. Phosphorylated RAFT1 was then incubated in a kinase reaction containing [γ-^{32}P]ATP and a GST fusion protein derived from amino acids 332–415 of p70^{S6k} that contains the RAFT1 phosphorylation site, Thr-389 (25). To avoid phosphorylation of GST-p70^{S6k} (332–415) by residual c-Abl, the Tyr-336 and Tyr-390 sites in GST-p70^{S6k} (332–415) were mutated to alanine (Tyr-Ala). The results demonstrate that preincubation of RAFT1 with kinase-active c-Abl inhibits autophosphorylation

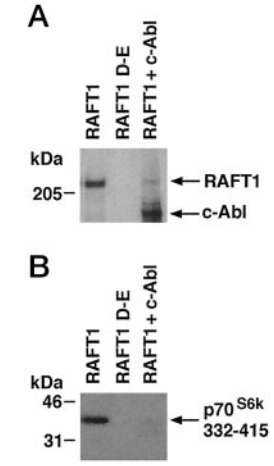

FIG. 5. **c-Abl inactivates RAFT1.** *A* and *B,* wild-type HA-RAFT1 or HA-RAFT1 D2357E mutant were transiently expressed in 293T cells. Lysates were subjected to immunoprecipitation with anti-HA. The immunopurified HA-RAFT1 and HA-RAFT1 D2357E were incubated in kinase buffer with cold ATP in the presence or absence of recombinant c-Abl. After the kinase reactions, the protein complexes were washed extensively and incubated with [γ-^{32}P]ATP in the absence (*A*) and presence (*B*) of GST-p70^{S6k} 332–415 Y336/390A mutant fusion protein. Phosphorylated proteins were resolved by SDS-PAGE and analyzed by autoradiography.

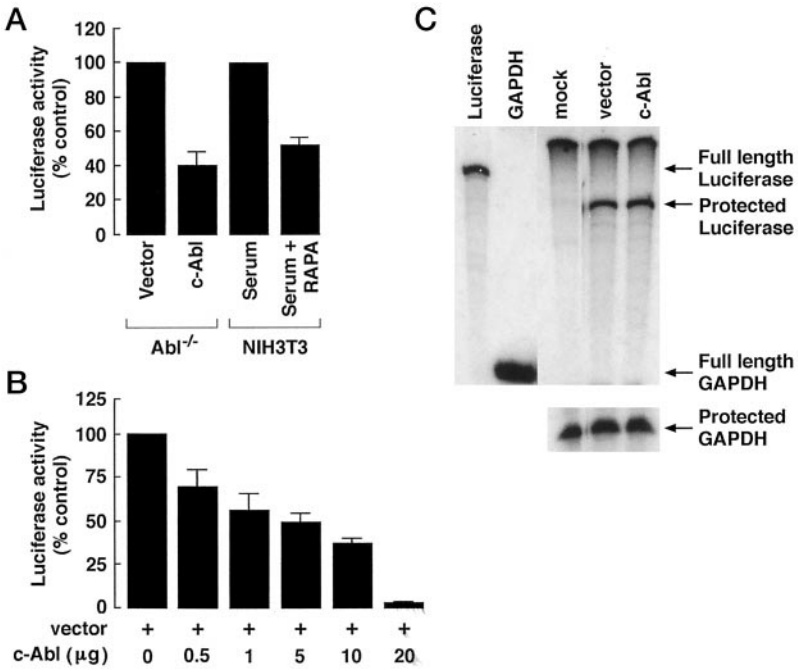

FIG. 6. **Effect of c-Abl on cap-dependent protein translation.** *A,* $Abl^{-/-}$ cells were transiently transfected with empty vector or c-Abl. Cells were cotransfected with 2 μg of pcDNA3-LUC-pol-CAT. Total cell lysates were prepared 24–36 h post-transfection and analyzed for luciferase activity. NIH3T3 cells were transfected with pcDNA3-LUC-pol-CAT. Twenty four h after transfection, cells were serum-starved for another 24 h. Rapamycin (50 ng/ml) and serum (10%) were added, and the cells were harvested after 4 h. Total cell extracts were prepared and assayed for luciferase activity. Luciferase activity is expressed as percent control (mean ± S.D of four independent experiments). *B,* 293T cells were transiently transfected with empty vector, pcDNA3-LUC-pol-CAT, and the indicated amounts of c-Abl. Total cell lysates were assayed for luciferase activity. Luciferase activity is expressed as percent control (mean ± S.D of four independent experiments). *C,* antisense GAPDH and luciferase probes (*lanes 1* and *2*) were tested against RNA from mock (*lane 3*), empty vector (*lane 4*), or c-Abl (*lane 5*) transfected cells. The positions of full-length probes and protected fragments are indicated on the *right.*

of RAFT1 (Fig. 5*A*). The results also demonstrate that c-Abl inhibits RAFT1-mediated phosphorylation of GST-$p70^{S6k}$ (*332–415*; Tyr-Ala) (Fig. 5*B*). Taken together, these findings demonstrate that c-Abl inhibits RAFT1 activity.

c-Abl Inhibits Cap-dependent Translation—Translation of cap-dependent mRNAs is inhibited by binding of unphosphorylated 4E-BP1 to eIF4E (27, 43). To determine whether c-Abl regulates cap-dependent translation, we transfected early passage embryo fibroblasts obtained from $Abl^{-/-}$ mice with the construct, pcDNA3-LUC-pol-CAT, that exhibits cap-dependent translation of luciferase (LUC) (44). Co-transfection of pcDNA3-LUC-pol-CAT and a vector expressing c-Abl blocked cap-dependent translation of LUC by 62% (Fig. 6*A*). Rapamycin treatment of NIH3T3 cells is associated with decreases in cap-dependent translation (27). To compare the magnitude of c-Abl-dependent inhibition of translation with that of rapamycin, NIH3T3 cells were serum-starved and transfected with pcDNA3-LUC-pol-CAT. Cap-dependent LUC activity was measured following treatment with 50 ng/ml rapamycin with and without serum. In concert with previous findings (27), rapamycin inhibited cap-dependent translation by 42% (Fig. 6*A*). Thus, inhibition of cap-dependent translation by c-Abl was comparable to that with rapamycin.

Recent studies have shown that RAFT1 phosphorylates 4E-BP1 on sites that regulate its interaction with the cap-binding protein eIF4E and thereby cap-dependent translation (25, 26). To assess further the effects of c-Abl on cap-dependent translation, we cotransfected 293T cells with pcDNA3-LUC-pol-CAT and increasing amounts of c-Abl. The results demonstrate that transfection of c-Abl is associated with a dose-dependent inhibition in LUC activity compared with that obtained in the absence of c-Abl transfection (Fig. 6*B*). To exclude the possibility that the effects observed reflect differences in mRNA concentration, RNase protection assays were performed on RNA extracted from parallel transfections using antisense GAPDH and luciferase probes. As expected, there were no protected fragments for the transfer RNA control (not shown), and only GAPDH mRNA was detected in mock-transfected cells (Fig. 6*C*). The luciferase/GAPDH mRNA ratio was 1.16 for c-Abl relative to vector (set at 1) (Fig. 6*C*). Taken together, these findings demonstrate that c-Abl inhibits RAFT1-mediated cap-dependent translation *in vivo*.

c-Abl Inhibits Phosphorylation of 4E-BP1—RAFT1-dependent phosphorylation of 4E-BP1 blocks the inhibitory interaction of 4E-BP1 with eIF4E and thereby induces cap-dependent translation (25, 26). To study the mechanism by which c-Abl inhibits cap-dependent translation, we asked whether c-Abl inhibits phosphorylation of 4E-BP1 *in vivo*. To address this issue, 293T cells were transfected with HA-4E-BP1 and increasing amounts of c-Abl. Phosphorylation of 4E-BP1 was assessed by band shifts in immunoblotting with anti-HA. The results demonstrate that expression of c-Abl significantly inhibits phosphorylation of 4E-BP1 (Fig. 7*A*). These findings demonstrate that c-Abl blocks cap-dependent translation by inhibiting RAFT1-mediated phosphorylation of 4E-BP1.

c-Abl Increases the Interaction of 4E-BP1 and eIF4E—As c-Abl inhibits RAFT1 activity, we asked whether c-Abl regulates the interaction of 4E-BP1 with eIF4E and thereby inhibits cap-dependent translation. Interactions of 4E-BP1 with eIF4E were assessed in fibroblasts from $Abl^{-/-}$ and wild-type mice. Cell lysates were incubated with a m^7GDP-coupled agarose resin. Bound proteins were eluted and analyzed by immunoblotting with anti-4E-BP1. As expected, eIF4E, which recognizes the cap structure, was associated with binding to the resin. 4E-BP1 was retained by the eIF4E-bound resin in fibroblasts from wild-type mice (Fig. 7*B, upper panel*). By contrast, there was little if any retention of 4E-BP1 when assaying lysates from $Abl^{-/-}$ fibroblasts (Fig. 7*B, upper panel*). As con-

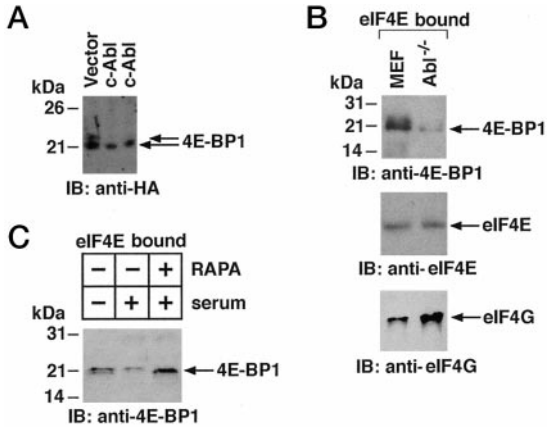

FIG. 7. **c-Abl inhibits phosphorylation of 4E-BP1 and interaction of 4E-BP1 with eIF4E.** *A,* 293T cells were transiently cotransfected with HA-4E-BP1 and 5 or 10 μg of pSRα-c-Abl. Total cell lysates were analyzed by immunoblotting (*IB*) with anti-HA. *B,* total lysates (20 μg) from Abl$^{-/-}$ and wild-type mouse embryo fibroblasts (MEF) were incubated with m^{7}GTP-agarose beads for 30 min at 25 °C. After washing, the proteins were eluted in sample buffer, resolved by SDS-PAGE, and transferred to nitrocellulose membranes. The filters were then analyzed by immunoblotting with anti-4E-BP1 (*upper panel*) or anti-eIF4E (*middle panel*). Total lysates from Abl$^{-/-}$ and wild-type mouse embryo fibroblasts were incubated with m^{7}GTP-agarose beads and analyzed by immunoblotting with anti-eIF4G (*lower panel*). *C,* NIH3T3 cells were serum-starved for 48 h. Cells were treated for 3 h with serum in the presence or absence of rapamycin (50 ng/ml). Total cell lysates were incubated with m^{7}GTP-agarose beads for 45 min at 25 °C. After incubation, the proteins were resolved by 15% SDS-PAGE and analyzed by immunoblotting with anti-4E-BP1.

trol, m^{7}GDP-bound proteins were eluted and also analyzed by immunoblotting with anti-eIF4E. The results demonstrate similar levels of eIF4E in lysates from wild-type and Abl$^{-/-}$ fibroblasts (Fig. 7*B, middle panel*). These findings indicate that c-Abl potentiates the interaction of 4E-BP1 with eIF4E. The eIF4F complex also includes two other subunits, eIF4G and eIF4A (45, 46). eIF4G serves as a modular scaffolding protein that binds eIF4E and regulates cap-dependent translation (45, 46). Since our results demonstrate that 4E-BP1 was retained by the eIF4E-bound resin in fibroblasts from wild-type mice, we asked whether the interaction of eIF4E with eIF4G is less in fibroblasts from wild type, to that compared with Abl$^{-/-}$, mice. Lysates were incubated with an m^{7}GDP-coupled agarose resin, and bound proteins were eluted and analyzed by immunoblotting with anti-eIF4G. The results demonstrate that, in contrast to fibroblasts from Abl$^{-/-}$ mice, the interaction between eIF4E with eIF4G was significantly less in fibroblasts from wild-type mice (Fig. 7*B, lower panel*).

To compare the magnitude of c-Abl-dependent induction of eIF4E·4E-BP1 complexes with that of rapamycin, NIH3T3 cells were serum-starved for 48 h. eIF4E-bound 4E-BP1 protein was determined following treatment with serum in the presence or absence of rapamycin. In concert with previous findings (27), the results demonstrate that addition of serum to starved cells is associated with inhibition of the interaction of 4E-BP1 with eIF4E and that rapamycin negates this effect (Fig. 7*C*). Taken together, these findings support a model in which c-Abl, like rapamycin, increases the interaction of 4E-BP1 with eIF4E and thereby inhibition of cap-dependent translation.

Inhibition of Protein Synthesis and Stimulation of the Interaction between 4E-BP1 and eIF4E in Response to IR—To determine the effect of IR on total protein synthesis, U-937 cells were serum-starved for 48 h and then incubated for 1 h in methionine-free media. Cells were exposed to IR in the presence of medium with serum containing [^{35}S]methionine and harvested at different times. The results demonstrate that IR exposure reduces protein synthesis by approximately half at 24 h (Fig. 8*A*). Effect of ionizing radiation on total protein synthesis was also assessed in fibroblasts from 4E-BP1$^{+/+}$ and 4E-BP1$^{-/-}$ mice (47). Fibroblasts from 4E-BP1$^{+/+}$ and 4E-BP1$^{-/-}$ mice were serum-starved for 48 h and then incubated for 1 h in methionine-free media. Cells were exposed to IR in the presence of medium with serum containing [^{35}S]methionine and harvested at different times. The results demonstrate that, by contrast to fibroblasts from 4E-BP1$^{-/-}$ mice, exposure of fibroblasts from 4E-BP1$^{+/+}$ with IR significantly inhibits total protein synthesis by 36 h (Fig. 8*B*).

To determine the functional interaction between c-Abl and RAFT1 *in vivo*, mouse embryo fibroblasts from Abl$^{-/-}$ and wild-type mice were exposed to IR and then harvested at various intervals. Total cell lysates were incubated with m^{7}GTP-coupled agarose resin. Bound proteins were eluted and analyzed by immunoblotting with anti-4E-BP1 and anti-eIF4E. The results demonstrate that, in contrast to Abl$^{-/-}$ fibroblasts, 4E-BP1 interacts constitutively with eIF4E in fibroblasts from wild-type mice (Fig. 9*A*, *upper panel*). The binding of 4E-BP1 and eIF4E was without significant effect on the total level of eIF4E (Fig. 9*A, middle panel*). Irradiation of wild-type, and not Abl$^{-/-}$, fibroblasts was associated with increases in binding of 4E-BP1 to eIF4E (Fig. 9*A, upper panel*). Furthermore, irradiation of wild-type, and not Abl$^{-/-}$, fibroblasts was associated with decreases in binding eIF4E to eIF4G (Fig. 9*A, lower panel*). To define the effects of IR on the interaction of eIF4E and 4E-BP1 in other cell types, NIH3T3 and U-937 cells were irradiated, and eIF4E-bound proteins were analyzed by immunoblotting with anti-4E-BP1. The results demonstrate that, as found in wild-type fibroblasts, binding of 4E-BP1 to eIF4E is increased in response to IR and that the kinetics of this association vary in different cell types (Fig. 9*B, upper panel* and data not shown). However, in this context, we have observed cell type variations in the time kinetics. Taken together, these findings support IR-induced inhibition of translation by c-Abl-mediated increases in the interaction of 4E-BP1 and eIF4E. Since our results demonstrate that exposure of cells to IR is associated with c-Abl-mediated inhibition in cap-dependent translation, we asked whether IR inhibits the interaction of eIF4E with eIF4G in U-937 cells. Lysates from cells exposed to IR were incubated with a m^{7}GDP-coupled agarose resin. Bound proteins were eluted and analyzed by immunoblotting with anti-eIF4G. The results demonstrate that IR induces a significant inhibition in the interaction of eIF4E with eIF4G (Fig. 9*B, bottom panel*). These findings indicate that exposure of cells to IR affects cap-dependent translation by inhibiting the interaction of eIF4E with eIF4G.

DISCUSSION

c-Abl Interacts with Multiple Members of the PIK Family—Yeast TOR1 and TOR2 and the mammalian mTOR (FRAP, RAFT1, and RAPT) are targets of the rapamycin·FKBP12 complex that controls translation initiation and G_1 phase progression (16, 19). The TOR proteins are members of a family of phosphatidylinositol kinase (PIK)-related kinases (18, 48). The PIK family also includes the p110 subunit of PI 3-kinase, DNA-PK$_{cs}$, ATM, ATR, and RAD3 (18, 44). Although homologous to the catalytic domains of kinases that phosphorylate PI and its derivatives, the TOR proteins, like DNA-PK$_{cs}$ and ATM, function as protein, rather than lipid, kinases. Members of the PIK family are involved in cell cycle control, DNA repair, and the DNA damage response. Few insights, however, are available regarding regulation of the catalytic function of PIK-related kinases. Recent studies have demonstrated that DNA-PK$_{cs}$ activates c-Abl in the response to DNA damage (2). In a potential feedback mechanism, c-Abl phosphorylates DNA-

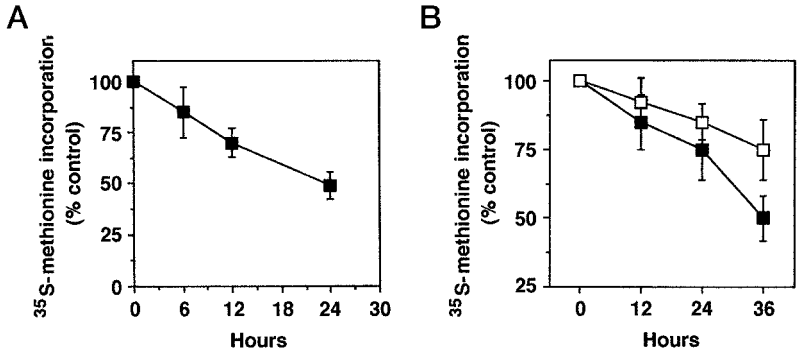

FIG. 8. **Ionizing radiation inhibits total protein synthesis in U-937 and 4E-BP1$^{+/+}$ fibroblasts.** U-937 (*A*) or fibroblasts from 4E-BP1$^{+/+}$ (■) and 4E-BP1$^{-/-}$ (□) mice (*B*) were serum-starved for 48 h and preincubated for 1 h in methionine-free medium. Serum was added with [^{35}S]methionine (100 μCi), and cells were exposed to 10 Gy IR. Cells were harvested at different times, and radioactivity incorporated into trichloroacetic acid-precipitable material was measured. The effect of IR was calculated as percent control and expressed as mean ± S.D of two to three independent experiments.

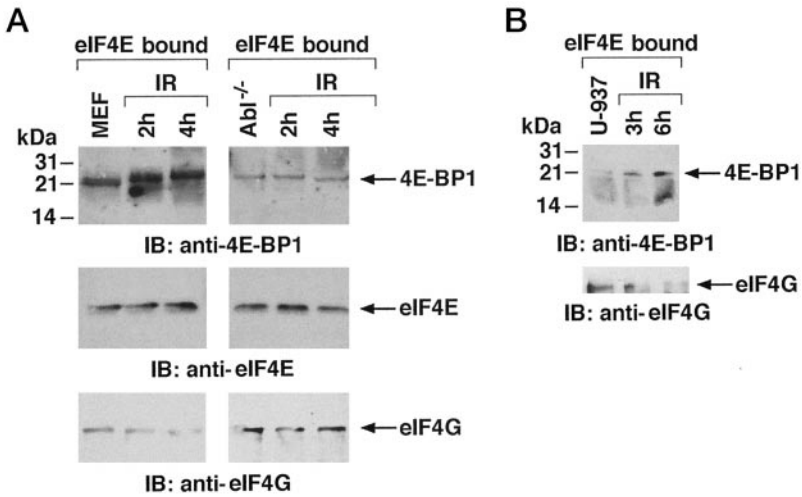

FIG. 9. **Ionizing radiation stimulates interaction of eIF4E with 4E-BP1.** *A,* fibroblasts from Abl$^{-/-}$ and wild-type mice were exposed to 20 Gy IR and harvested at the indicated times. Total cell lysates (10 μg) were incubated with m^{7}GTP-agarose beads for 45 min at 25 °C. After incubation, the proteins were resolved by SDS-PAGE and analyzed by immunoblotting (*IB*) with anti-4E-BP1 (*upper panel*), anti-eIF4E (*middle panel*), or anti-eIF4G (*lower panel*). *B,* U-937 cells were exposed to 20 Gy IR and harvested at the indicated times. Total cell lysates were incubated with m^{7}GTP-agarose beads and analyzed by immunoblotting with anti-4E-BP1 (*upper panel*) or anti-eIF4G (*lower panel*).

PK_{cs} and inhibits DNA-PK activity (2). ATM also associates with c-Abl (8–9). Whereas phosphorylation of c-Abl by ATM results in the activation of c-Abl, it is not known if c-Abl regulates ATM activity. Other work has shown that c-Abl binds constitutively to PI 3-kinase in cells (14). Activation of c-Abl by DNA damage is associated with phosphorylation of the p85 subunit of PI 3-kinase. Moreover, phosphorylation of p85 by c-Abl inhibits PI 3-kinase activity *in vitro* and in cells exposed to DNA-damaging agents (14). Collectively, these findings demonstrate that c-Abl interacts with multiple members of the PIK family and negatively regulates DNA-PK_{cs} and PI 3-kinase in the response to DNA damage. The present work extends the relationship between c-Abl and PIK family members with the demonstration that c-Abl binds directly to RAFT1. The results demonstrate that c-Abl phosphorylates RAFT1 and thereby inhibits RAFT1 activity.

RAFT1-dependent Regulation of Cap-dependent Translation and G_1 Progression—The rapamycin·FKBP12 complex binds directly to RAFT1 (16, 19, 20). In yeast, rapamycin inhibits translation by >90% (49), whereas in mammalian cells this agent causes a partial inhibition (50–52). Also, cells treated with rapamycin or deficient in TOR proteins arrest growth in G_1 phase of the cell cycle (49). The cell cycle arrest associated with inhibition or loss of RAFT1 activity has been attributed to a secondary effect of the down-regulation of translation (49). TOR functions upstream to activation of $p70^{S6}$ kinase and 4E-BP1 (53, 54). Activity of the $p70^{S6}$ kinase may be necessary for the G_1-S phase transition in certain cells (55, 56). The translation of mRNAs with terminal oligopyrimidine tracts is mediated in part by $p70^{S6}$ kinase (57–59). 4E-BP1, by contrast, interacts with the mRNA 5′ cap-binding protein, eIF4E, and thereby inhibits cap-dependent translation (33). The translation of most eukaryotic mRNAs is mediated by cap-dependent mechanisms (60). Thus, inhibition of RAFT1 by rapamycin contributes to translational arrest by down-regulation of $p70^{S6}$ kinase and by increasing the affinity of 4E-BP1 for eIF4E (26, 53).

Other than the rapamycin·FKBP12 complex, there are no known inhibitors of RAFT1 activity. The present results demonstrate that c-Abl phosphorylates RAFT1 and thereby down-regulates RAFT1 activity. The inhibitory effects of c-Abl on RAFT1 were at least as pronounced as those reported for rapamycin. The findings of the present study in Abl$^{-/-}$ cells support a function for c-Abl in inhibiting RAFT1 activity and cap-dependent translation. Indeed, the c-Abl$^{-/-}$ cells, but not their c-Abl$^{+/+}$ counterparts, proliferate more rapidly (data not shown). c-Abl is activated in the response of cells to DNA damage (1–2). The findings that irradiation increases binding of 4E-BP1 to eIF4E and that this effect is c-Abl-dependent supports a model in which DNA damage-induced activation of

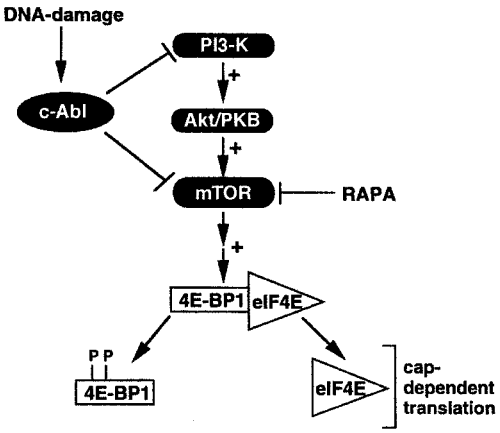

FIG. 10. **Schematic model of c-Abl-mediated regulation of the mTOR/RAFT1/FRAP cascade and 5′ cap-dependent translation.**

c-Abl results in inhibition of RAFT1 and thereby dephosphorylation of 4E-BP1. Down-regulation of cap-dependent translation can contribute to arrest of cell cycle progression by inhibiting expression of mitogen-sensitive mRNAs (*e.g.* c-myc and ornithine decarboxylase) or unstable G_1 cyclins. Significantly, previous studies have demonstrated that overexpression of c-Abl in fibroblasts induces G_1 arrest (42, 61, 62). Expression of c-Abl in *Schizosaccharomyces pombe* similarly induces growth arrest by a mechanism dependent on the c-Abl kinase function (63). In one study using fibroblasts with disruption of the p53 or retinoblastoma genes, c-Abl-dependent growth arrest was found to be dependent on p53, but not retinoblastoma (64). Other work has shown that growth arrest induced by c-Abl requires both p53 and retinoblastoma (65). The present finding that c-Abl inhibits RAFT1 supports the existence of alternative translation-dependent mechanisms by which c-Abl could contribute to G_1 growth arrest.

Regulation of Translation in the Genotoxic Stress Response—The response of cells to genotoxic stress includes cell cycle arrest. Little is known about the effects of DNA damage on translation and how such effects contribute to the genotoxic stress response. Levels of certain proteins, such as p53, increase in cells after treatment with genotoxic agents (66). IR-induced increases in p53 levels have been attributed in part to enhanced translation of p53 mRNA (67). In addition, the p53 3′-untranslated region functions in both repression and activation of translation in the IR response (67). The present finding that c-Abl inhibits RAFT1 activity provides support for a model in which genotoxic stress induces inhibition of cap-dependent translation. Indeed, our results show that IR treatment is associated with over 50% inhibition of protein synthesis. Similar findings have been obtained in mammalian cells treated with rapamycin (27). Inhibition of translation in the response to DNA damage could contribute to cell cycle arrest. In this context, rapamycin inhibits translation of the insulin-like growth factor II which functions in promoting survival (68–70). Thus, c-Abl-mediated inhibition of RAFT1 and induction of the interaction between 4E-BP1 and eIF4E could, like the effects of rapamycin, contribute to cell cycle arrest.

Recent study has shown that c-Abl inhibits the lipid kinase function of PI 3-kinase and thus supports the inhibition of survival pathway (71). Inhibition of PI 3-kinase has been linked to the down-regulation of PI-(3,4,5)P_3 (72, 73). PI-(3,4,5)P_3 binds to the pleckstrin homology domain of Akt (PKB) and facilitates activation of Akt through phosphorylation by the 3-phosphoinositide-dependent protein kinases 1 and 2 (74–76). Significantly, Akt functions upstream to the activation of RAFT1 (77). Thus, 4E-BP1 is phosphorylated and inactivated by Akt-mediated signaling (77). Taken together, the findings that c-Abl inhibits both PI 3-kinase and RAFT1 (Fig. 10) support a model in which down-regulation of the PI 3-kinase/Akt/RAFT1 cascade and thereby cap-dependent translation may contribute to the stress response to genotoxic agents.

Acknowledgments—We thank Ruibau Ren, David Baltimore, and Young Whang for providing mouse embryo fibroblasts from wild-type and $Abl^{-/-}$ mice; and Perry Blackshear for providing mouse embryo fibroblasts from 4E-$BP1^{+/+}$ and 4E-$BP1^{-/-}$ mice. We also thank John Blenis for critical reading of the manuscript and valuable suggestions.

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