Adenovirus type 2 (Ad2) early region 4 ORF4 (E4orf4) triggers a major death pathway that requires its accumulation in cellular membranes and its tyrosine phosphorylation. This program is regulated by Src family kinases and triggers a potent ZVAD (benzoylcarbonyl-Val-Asp-Asp-Asp-fluoromethylketone) and Bcl2-resistant cell death response in human-transformed cells. How E4orf4 deregulates Src-dependent signaling is unknown. Here we provide strong evidence that a physical interaction requiring the kinase domain of Src and the arginine-rich motif of E4orf4 is involved. The Src binding domain of E4orf4 overlaps with, but is distinct from that of the B4 subunit of protein phosphatase 2A (PP2A-B4) and some E4orf4 complexes contain both PP2A and Src. Functional assays using mutant E4orf4 revealed that deregulation of Src signaling, activation of the Jun kinase pathway, and cell blebbing were all critically dependent on Src binding. In contrast, PP2A-B4 binding per se was not required to engage the Src-dependent death pathway but was more critical for triggering a distinct death activity. Both E4orf4 death activities were manifested within a given cell population, were typified by distinct morphological features, and contributed to overall cell killing, although to different extents in various cell types. We conclude that E4orf4 binding to the Src kinase domain leads to deregulation of Src signaling and plays a crucial role in induction of the cytoplasmic death pathway. Nonetheless, both Src and PP2A enzymes are critical targets of E4orf4 that likely cooperate to trigger E4orf4-induced tumor cell killing and whose relative contributions may vary in function of the cellular background.

The adenovirus type 2 (Ad2)1 early region 4 ORF 4 (E4orf4) is a 14-kDa early viral gene product whose function remains ill-defined. E4orf4 is not essential for viral growth, although several activities were reported during viral infection that were linked to its ability to interact with cellular PP2A. However, the exact consequence on PP2A activity remains unclear (1–6). It is believed but not proven that E4orf4 cooperates with other viral products to trigger cell death at the end of the infectious cycle to propagate viral progeny (7). In mammalian cells, over-expression of E4orf4 triggers p53-independent cell death, and evidence accumulates that E4orf4 killing is much higher in transformed and cancer cells (7–11). Because, adenoviral infection drives a process similar to cell transformation by stimulating proliferation and inhibiting p53-dependent cell death, it is conceivable that E4orf4 has evolved to act in a transformed-like genetic background. Deciphering the mechanisms involved in cell death induction is of great interest, because they may unravel ways to manipulate oncogene signaling to trigger tumor-selective death programs.

Emerging evidence suggests that E4orf4 has several activities, some of which are cell type-specific. The specific localization of E4orf4 to the cytoplasm, cytoskeleton, and the nucleus illustrates the complexity of E4orf4 signaling with a number of potentially important cellular targets (12, 13). Caspase-dependent apoptosis was observed in specific cell lines, but E4orf4-induced cell death generally proceeds independently of the classical pathways for apoptosis induction (death receptor and mitochondrial pathways) (14, 15). Indeed in most transformed and cancer cells, E4orf4 triggers a ZVAD (benzoylcarbonyl-Val-Asp-Asp-Asp-fluoromethylketone) and Bcl2-resistant cell death pathway associated with some features of apoptosis, including externalization of phosphatidylserines, cell and nuclear shrinkage, chromatin condensation, and phagocytosis by neighboring cells (8, 12, 15).2 We have shown that this cell death pathway is associated with E4orf4 accumulation in the cell membrane-cytoskeleton and is first manifested by the appearance of dramatic actin changes leading to dramatic cell blebbing (12). This so-called cytoplasmic death activity is regulated by Src tyrosine kinases, requires the tyrosine phosphorylation of E4orf4, and involves the calcium-regulated cysteine protease calpains (13, 15). Importantly, we have shown that E4orf4 targeting to cell membranes recapitulates the Src-dependent death activity (15), suggesting that tyrosine-phosphorylated E4orf4 acts by disrupting some critical tyrosine kinase signaling pathway on membranes. A distinct death activity was also observed, in the absence of E4orf4 tyrosine phosphorylation (15). This death program was rather associated with E4orf4 accumulation in the cell nucleus and led to a distinct morphological phenotype, characterized by dramatic cell shrinkage in the absence of early blebbling induction. The nuclear targets of E4orf4 remain...
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unknown, but may be related to its ability to trigger an irreversible GlcM growth arrest in yeasts and in specific cancer cell lines (16, 17). Notably, E4orf4 killing and growth inhibition was shown to depend on its ability to bind to the regulatory subunit Βα of protein phosphatase 2A (PP2A-Βα) and recruit PP2A phosphatase activity (10, 16–18). Whether E4orf4 acts by inhibiting and/or stimulating PP2A activity toward specific substrates in a way that relies on recruitment to PP2A-Βα to specific cell compartments remains unclear. Whatever the case, these studies indicate that extra functions are required for cell killing and that some PP2A-independent killing also exists. Although it is clear that E4orf4 possesses distinct activities in various cell types, the functional relationship between Src and PP2A was not addressed, and the molecular mechanisms involved in engagement of the so-called cytoplasmic death pathway remain elusive.

We have delineated the determinants of E4orf4-Src association at the molecular level. E4orf4 mutant proteins were used to address the role of Src and PP2A-Βα binding in induction of the cytoplasmic death pathway typified by early changes in actin dynamics. The results strongly suggest that a physical interaction with the kinase domain of Src-like tyrosine kinases is critical to activate E4orf4 cytoplasmic death functions. This interaction requires a highly basic motif on E4orf4 that overlaps with, but is distinct from, the PP2A-Βα binding site. Based on the available data, E4orf4 binding to PP2A-Βα per se is dispensable for deregulation of Src signaling and activation of the cytoplasmic death pathway and appears more critical for induction of a distinct death program.

MATERIALS AND METHODS

Expression Vectors and Mutagenesis—The following expression vectors were described previously: Ad2 HA-E4orf4 (8); FLAG-E4orf4 (13); Myc-E4orf4 (12); FLAG-E4orf4-green-fluorescent protein (GFP) and FLAG-GFP-NSL (15); GST-SH2-βv and GST-SH3-βv (designated SH2v and SH3v) and Myc-p130Cas from Dr. Michel Tremblay, McGill University, Montreal, Canada (19, 20); GST-c-Src, GST-SH2c-Src, and SH2v and SH3v) and Myc-p130Cas from Dr. Michel Tremblay, McGill University, respectively, both supplemented with 10% fetal bovine serum and 25 mM HEPES, pH 7.4, 10 mM MgCl2, and 0.5 mM CaCl2, fixed in 3.7% formaldehyde/CO2/thermoregulated chamber and a 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, Molecular Probes), and staining of DNA was performed using 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, Molecular Probes). Long-term cell killing was determined by colony-forming assays as described (13). When indicated, the broad spectrum caspase inhibitor Boc-Asp(OMe)-CH2-pAb (Boc-D-fmk, Calbiochem) was added to the culture medium at 15 μM during transfection and at 30 μM after transfection. For every 24 h, 0.85 NA objective lens. The blebbing inducing activity and chromatin condensation were determined (see above). Briefly, cells were gently washed in PBS containing 1 mm MgCl2 and 1 mm CaCl2, fixed in 3.7% formaldehyde/PBS for 20 min, and permeabilized in 0.2% Triton X-100/PBS for 5 min. Immunodetection of HA-E4orf4, E4orf4-GFP, and untagged E4orf4 was performed using mouse HA.11 anti-HA antibody (Sigma-Aldrich), rabbit anti-GFP (Clontech), or rabbit 2419 anti-E4orf4, respectively, followed by Alexa 594- or Alexa 488-labeled goat anti-rabbit IgG (Molecular Probes), and staining of DNA was performed using 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, Molecular Probes). Long-term cell killing was determined by colony-forming assays as described (13).

GST Fusion Proteins and in Vitro Binding Assays—GST-c-Src constructs were introduced in Escherichia coli BL21 strain expressing GroES and GroEL chaperones encoded by preP4-GroESL vector (a generous gift from Drs. Kurt Amrein and Martin Stieger, Roche Research Center, Hoffmann-La Roche Inc., Nutley, NJ) (28). The fusion products were produced by growing bacterial cultures to an OD600 of 1.5 at 37 °C, and the beads were washed three times in washing buffer (PBS containing 1 Triton X-100 and 1 mM EDTA). To remove the bound chaperones, beads were incubated in ATP buffer (50 mM Tris-HCl, pH 7.4, 10 mM MgSO4, and 2 mM ATP) for 20 min at 37 °C, washed three times in washing buffer, and used for in vitro binding assays. The
amount of purified proteins was evaluated on a Coomassie Blue-stained gel. In vitro binding assays were performed as described (13) by incubating equal amounts of transfected total cell lysates (1 mg) with 5 μg of freshly prepared immobilized GST fusion proteins overnight at 4 °C.

The beads were recovered by centrifugation, washed once in lysing buffer, then twice in lysis buffer containing only 0.1% Nonidet P-40, and boiled in sodium dodecyl sulfate (SDS) sample buffer. Bound proteins were analyzed by Western blot analysis with the appropriate antibody. The GST-FLAG-E4orf4 was produced as described (12). The GST moiety was removed by incubating the beads in protease buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 4 units of PreScission Protease™ (Amersham Biosciences) overnight at 4 °C with rotation. Recombinant FLAG-E4orf4 protein was recovered in the supernatant, and direct binding to c-Src kinase domain was performed using 5 μg of the indicated GST-c-Src proteins and 200 ng of purified FLAG-E4orf4 protein, as described above. In vitro kinase assays were performed using 250 ng of bound GST-c-Src and 2.5 μg of purified FLAG-E4orf4 or GST in kinase buffer (50 mM Hepes pH 7.4, 10 mM MgCl₂, 0.1% β-mercaptoethanol, 0.1 mM Na₂VO₄ containing 0.1 mM ATP and 10 μCi of [γ-³²P]ATP in a 50-μl reaction mixture. The reactions were allowed to proceed for 20 min at 30 °C and stopped by adding 25 μl of 3x SDS sample buffer. Labeled samples were resolved on SDS-PAGE and visualized by autoradiography or analyzed by Western blot after electrophoresis on nitrocellulose.

Immunoprecipitation, Western Blotting, and Antibodies—For coprecipitations, cells on 10-cm plates were lysed with 0.5 ml of modified radioimmunoprecipitation assay buffer (RIPA: 50 mM Hepes pH 7.4, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 10% glycerol, 50 mM NaF, 10 mM β-glycerophosphate, 1 mM Na₂VO₄, 15 μg/ml leupeptin, 5 μg/ml aproptin, 1 μg/ml pepstatin A), and cytoskeletal proteins were immediately diluted with 0.5 ml of HNTG buffer (50 mM Hepes pH 7.4, 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol), as described (29). The lysates were incubated with protein G-Sepharose (Amersham Biosciences) or protein G Plus-agarose (Oncogene Research Products) for 20 min on ice and cleared by centrifugation. Immunoprecipitation was carried out with mouse anti-FLAG M2 (Sigma-Aldrich), rabbit SRC2 anti-Src (Santa Cruz Biotechnology), mouse Ab1 anti-Src (Calbiochem), mAb 327 anti-c-Src (26), or rabbit SRC2 anti-Src (Santa Cruz Biotechnology) antibodies over night at 4 °C. Immune complexes were collected on protein G-Sepharose or protein G Plus-agarose and washed three times in modified RIPA containing only 1% Triton X-100 before analysis on SDS-PAGE. Equal amounts of immune complexes were resolved on 9 or 11% SDS-PAGE, transferred to nitrocellulose, and probed for immunoblotting. To disrupt the antigen-antibody complex before reprobing, immobiloblotts were incubated in stripping buffer (62.5 mM Tris-HCl pH 6.7, 2% SDS, 100 mM β-mercaptoethanol) for 30 min at 60 °C, washed in PBS-Tween (0.1%) at room temperature and reprobed for immunoblotting. The following antibodies were used for immunoblotting analyses: mouse anti-cortactin (4F11, Upstate Biotechnology); mouse anti-FLAG (M2, Sigma); mouse anti-HA (HA.11, Babco); mouse anti-Myc (9E10, Sigma); mouse anti-poliovirus middle T-antigen (Pab 762) (30); mouse anti-phosphotyrosine (RC20:HRPO, Transduction Laboratories); rabbit anti-Src (SRC2, Santa Cruz Biotechnology); mouse anti-phospho-Src (Tyr416) (Cell Signaling, NEB), mouse anti-FPS2A-Ca (clone46, BD Biosciences), rabbit anti-phospho-p-38 (Thr180/Tyr182) (Cell Signaling, NEB), rabbit anti-ERK2 (clone46, BD Biosciences), rabbit anti-phospho-p44/42 MAPK (Thr202/Tyr204) (Cell Signaling, NEB), rabbit anti-ERK2 (clone86, Babco), mouse E10 anti-phospho-p44/42 MAPK (Thr202/Tyr204) (Cell Signaling, NEB), antibody was analyzed by Western blot using anti-FLAG M2, anti-mT (Pab762), and anti-Myc (9E10) antibodies. The input lanes represent 1% of the total cell lysates (TL). Results are representative of three independent experiments. SH3c, v-Src SH3 domain; SH3e, c-Src SH3 domain; SH2c, c-Src SH2 domain; SH2e, v-Src SH2 domain; SH1c, c-Src SH2 domain; SH1, c-Src kinase domain. B, 283T cells were transfected with FLAG-E4orf4 together with the indicated c-Src constructs. Cell lysates were prepared from 283T cells transfected with FLAG-E4orf4, FLAG-GFP, mT, Myc-p130Cas, or Myc-FAK plus c-Src (Y327F), which were incubated with the indicated immobilized GST-c-Src fusion proteins, as depicted. The retained ma-
bit H-79 anti-c-Jun (Santa Cruz Biotechnology), rabbit anti-phospho-c-Jun (Ser\(^{63}\)) II (Cell Signaling, NEB), mouse anti-\(\beta\)-actin (AC-74, Sigma-Aldrich), and rabbit 2419 anti-E4orf4. Rabbit 2419, 2418, and 2420 anti-E4orf4 antibodies were described (12). The anti-phospho-E4orf4 (Tyr\(^{42}\)) was produced by injecting rabbits with a chemically synthesized peptide comprising the phosphorylated Tyr\(^{42}\) (HEGVY\([\text{PO}_3\text{H}_2]\)-IEPEARGRLC), which was coupled to mcKLH (Imject Mariculture, Pierce) following the manufacturer’s recommendations. The serum was purified against the immobilized non-phosphorylated peptide (HEGVYIEPEARGRLC), was absorbed on immobilized phosphorylated peptide using the Sulfolink Kit (Pierce), and was finally purified against phosphotyrosines linked to agarose (O-phospho-t-tyrosine-agarose, Sigma-Aldrich) according to the manufacturer’s recommendations. The specificity of the resulting anti-phospho-E4orf4 (Tyr\(^{42}\)) was tested by Western blot analysis of E4orf4 immune complexes and total cell lysates from 293T cells transfected with wild-type FLAG-E4orf4, compared with mutant FLAG-E4orf4 (Y42F) alone or together with c-Src or v-Src to induce maximum tyrosine phosphorylation of Ad2 E4orf4, as described (13). The antibody reacted specifically with wild-type Ad2 E4orf4 but not with mutant E4orf4 (Y42F), and the specific signal was proportional to the level of tyrosine phosphorylation.

RESULTS

Ad2 E4orf4 Directly Interacts with the Kinase Domain of Src—In several transformed and cancer cell lines expressing Ad2 E4orf4, including 293T, C-33A, and H1299, a membra-
ne-cytoskeleton fraction of E4orf4 is associated with Src family kinases, and inhibition of Src kinases interferes with cell death induction (12, 13, 15). To obtain further insight into how E4orf4 usurs Src kinases to trigger cell death, we sought to analyze the molecular determinants of E4orf4-Src interaction. To identify the interaction site on Src kinases, we produced functional recombinant GST fusion proteins containing the Src homology domain (SH) 1, SH2, SH3, or bearing deletions of c-Src regulatory sequences (\(\Delta\)SH), which is the prototype of the ubiquitously expressed cellular Src kinases, Src, Fyn, and Yes (reviewed in Ref. 32). In vitro binding assays were performed using cell lysates from 293T transfected with FLAG-E4orf4 or FLAG-GFP as a negative control for Src binding. Lysates from 293T transfected with polyomavirus middle T-antigen, FAK, or p130Cas were used as positive controls for binding to Src kinase domain (SH1), SH2, and SH3 domains, respectively. We found that c-Src kinase domain (SH1), but not the SH2 and SH3 domains, is required for E4orf4 binding (Fig. 1A). Coinmunoprecipitations using lysates from 293T cotransfected with FLAG-E4orf4 and c-Src mutants confirmed that Ad2 E4orf4 interacts with the kinase domain in vivo (Fig. 1B). Interestingly, significant E4orf4 binding to v-Src SH3 domain, but not c-Src SH3 domain, was also detected as previously reported for FAK (Fig. 1A, myc-FAK, left panel). Such an interaction is believed to facilitate a v-Src-FAK interaction in more invasive cells, which is cell adhesion- and SH2-independent. This interaction relies on residues in the RT loop region of the v-Src SH3 domain that differ from those of the c-Src SH3 domain (33, 34). The N-terminal sequence of Ad2 E4orf4 contains a proline-rich motif, PALPAPP, conforming to a v-Src SH3 binding consensus (PXXP\(\Phi\)XX where \(\Phi\) is a hydrophobic residue) (35). However, deletion of the E4orf4 Pro-rich motif did not ablate binding to Src kinases (data not shown). Consistently, deletion of c-Src SH3 had no effect, whereas deletion of the kinase domain \(\Delta\)SH1c) ablated binding to c-Src in vitro (Fig. 1A). A recombinant GST-E4orf4 readily bound to full-length GST-c-Src and to GST-kinase\(^{\text{Src}}\), but not to the kinase domain-deleted GST-c-Src \(\Delta\)SH1c) or to GST alone, indicating that the interaction is direct (Fig. 1C). Furthermore, GST-c-Src could mediate the phosphorylation of recombinant E4orf4 in vitro (Fig. 1C, right panel). Immunoblot analysis of the in vitro-phosphorylated E4orf4, using a phosphospecific E4orf4 (Tyr\(^{42}\)) antibody raised against the major phosphorylated tyrosine in vivo (13) confirmed the specificity of phosphorylation. Altogether, the data provide strong support for a physical interaction between Ad2 E4orf4 and Src family kinases that primarily involves sequences residing within the kinase domain and may contribute to tyrosine phosphorylation of Ad2 E4orf4 in vivo.

Binding to Src Kinase Domain Is Mediated by a Core Arg-rich Motif, Which Overlaps With but Is Distinct from the PP2A-B\(a\) Binding Site—Preliminary experiments using Ad2 E4orf4 deletion mutants indicated that the C-terminal half of E4orf4 (amino acids 64–114), but not the N-terminal portion (amino acids 1–63) interacts with GST-c-Src in vitro (data not shown). To delineate the E4orf4 sequences involved, we used mutant proteins containing Ala substitutions in the C-terminal half of Ad2 E4orf4, which were previously characterized for their association with PP2A-B\(a\) in vivo (Fig. 2A) (18). In vitro binding assays were performed using GST-c-Src, GST-kinase\(^{\text{Src}}\).
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Fig. 3. E4orf4 association with c-Src in vivo is mediated by the Arg-rich motif and does not require PP2A-Ba binding. A, equal amounts of 293T cell lysates transfected with the indicated E4orf4 constructs or with the vector alone (EV) were processed for immunoprecipitation (IP) of endogenous c-Src using mab327 anti-c-Src. Immune complexes were analyzed by Western blot with anti-E4orf4 (2419) and anti-c-Src, and the amounts of transfected E4orf4 proteins are shown in total cell lysates (TL). B, immune complexes of E4orf4 were isolated from 293T cotransfected with the indicated E4orf4 constructs and FLAG-PP2A-Ba and were analyzed by Western blot using anti-FLAG (M2) and anti-E4orf4 (2419). The amounts of transfected E4orf4 proteins and PP2A-Ba in total cell lysates are shown (TL). Relative binding to endogenous c-Src (A) or exogenous PP2A-Ba (B) was estimated from scanned-derived images by densitometric analysis with Quantity One® software, and the percent binding is expressed relative to the binding of wild-type E4orf4 (100%). The data are the means ± S.D. of at least three independent experiments performed with untagged E4orf4 or FLAG-E4orf4 proteins (A) or performed with HA-E4orf4 and untagged E4orf4 proteins (B).

(SH1), or kinase-deleted GST-c-Src (∆SH1), and lysates from 293T expressing equivalent amounts of the wild-type or mutant E4orf4 proteins. We found that mutation of a cluster of Arg residues spanning residues 69–75 dramatically affected E4orf4 binding to c-Src kinase domain. Mutation of Arg74/75/79 drastically decreased E4orf4 binding to kinasec-Src, and mutation of the whole cluster (Arg69/70/72/73/74/75, hereafter named 6R-A) completely ablated the in vitro interaction (Fig. 2B). In contrast, significant binding remained when Phe84 or Arg81/Phe84 were substituted for Ala residues (80 to 60% residual binding). These mutations were used to inhibit PP2A-Ba binding to E4orf4 (18) (see Fig. 3B), suggesting that direct binding to c-Src was largely independent of PP2A-Ba, at least when an excess of c-Src, or kinasec-Src were present. Identical results were obtained using either N-terminal-tagged, or -untagged versions of E4orf4 proteins. To further delineate the minimal sequence requirement for binding to kinasec-Src in vitro, GFP fusion proteins containing the Arg core motif alone (amino acids 62–79) or including some critical determinants for PP2A binding (amino acids 62–95) were expressed in 293T, and binding was assessed by pull-down assays. Although the minimal core Arg motif was sufficient to drive specific binding to the kinasec-Src, the extended region provided more efficient binding (Fig. 2C). These results suggest that direct binding of E4orf4 to the kinase domain of c-Src is primarily mediated by the Arg-rich motif (62–75) and that some sequences downstream (80–95) stabilize the interaction.

To address the molecular determinants of Src binding under more physiological conditions, coimmunoprecipitations with endogenous c-Src were performed in 293T cells expressing the wild-type or mutant untagged E4orf4 proteins. Coexpression of FLAG-PP2A-Ba with E4orf4 proteins was also performed to compare the ability of the mutant proteins to associate with PP2A-Ba in the presence of unlimited amounts of PP2A-Ba. As shown in Fig. 3A, mutation of the minimal Arg-rich motif (6R-A) completely abolished E4orf4 association with endogenous c-Src, consistent with loss of binding to kinasec-Src in vitro (Fig. 2B). Such mutations did not completely ablate binding to exogenous PP2A-Ba, but decreased it by 40% relative to wild-type E4orf4 binding (Fig. 3B). In marked contrast, mutation of either Arg81/Phe84 or Phe84 alone did not markedly affect E4orf4 association with endogenous c-Src (88 to 78% residual binding). However, E4orf4 association with exogenous PP2A-Ba was completely inhibited, as previously reported (18). The results strongly suggest that the intrinsic ability to bind to PP2A-Ba is not required to mediate E4orf4 association with Src in vivo, which rather depends on the ability of E4orf4 to physically interact with the kinase domain of c-Src in vitro.

Given that Src and PP2A associations are mediated by overlapping sequences on E4orf4, we attempted to determine whether the binding of Src affects the binding of PP2A-Ba to c-Src in vitro or vice versa. E4orf4 was coexpressed with large amounts of v-Src, and the recruitment of endogenous PP2A-C (PP2A catalytic subunit) by E4orf4 was evaluated by coprecipitations. In principle, interfering with PP2A-Ba binding should also interfere with the recruitment of PP2A-C. E4orf4 association with PP2A-C was not decreased and on the contrary was slightly promoted by v-Src. A similar effect was observed upon overexpressing PP2A-Ba (Fig. 4A). Given that Src and PP2A interact with one another in other systems (36–40), the recruitment of PP2A-C could be mediated by v-Src in this particular experiment. As another approach, we compared the ability of endogenous c-Src to associate with PP2A-C in the presence and absence of E4orf4. We repeatedly observed increased PP2A-C association with active c-Src in the presence of E4orf4 (Fig. 4B). This suggests that E4orf4 binding to both PP2A-Ba and c-Src exists, because increased Src binding to PP2A-C was likely mediated by the ability of E4orf4 to recruit PP2A holoenzyme by specific binding to the Bo subunit. Whatever the case, the data indicated that binding to Src and PP2A was not competitive. Reciprocally, overexpression of PP2A-Ba did not inhibit E4orf4 association with endogenous c-Src, and no significant competition for E4orf4 binding was observed, considering that overexpression of PP2A-Ba inhibited E4orf4 expression in cotransfection experiments (Fig. 4C). Altogether, the data suggest that at least part of the E4orf4 signaling complexes contain both c-Src and PP2A and that some cooperative signaling may exist in the context of the wild-type E4orf4 protein.

E4orf4 Binding to the Kinase Domain of Src Is Involved in Modulation of Src-dependent Phosphorylation and Activation of the JNK Pathway—E4orf4 expression leads to increased Src-dependent phosphorylation of specific target proteins, including the F-actin-binding protein cortactin (12). Cortactin associates with E4orf4, along with other unidentified tyrosine-phosphorylated proteins (13). To determine whether E4orf4 interaction with kinasec-Src can activate the cytoplasmic function of E4orf4 in vivo, we looked at whether E4orf4 could promote cortactin association with a mutant Src protein containing only the kinase domain and C-terminal regulatory sequences. Cortactin normally associates with Src in a SH2-dependent way and cannot be recruited by kinasec-Src (41–43). The kinase domain of c-Src was overexpressed alone or together with E4orf4 in 293T cells, and immunoprecipitations...
were performed using an antibody directed to the C terminus of Src proteins (SRC2). When kinasec-Src was overexpressed alone, only few phosphotyrosine-containing proteins were detected in immune complexes, and there was no increase in cortactin relative to control cells (Fig. 5A, lane 2), suggesting that the small amount of cortactin was coprecipitated with endogenous Src kinases (Fig. 5A, lane 1). When E4orf4 was coexpressed with kinasec-Src, a marked increase in cortactin level and in the number of tyrosine-phosphorylated proteins was detected in immune complexes of kinasec-Src (Fig. 5A, lane 4). Immunoprecipitation with anti-SRC2 was not efficient enough to detect E4orf4-dependent recruitment of cortactin and tyrosine-phosphorylated proteins to endogenous Src kinases (lane 3), because E4orf4 associates with less than 1–5% of total endogenous Src proteins, depending on the cell types (12). Thus the data strongly suggest that E4orf4 binding to kinasec-Src is sufficient to support at least some of the active E4orf4-Src signaling complexes. Indeed, overexpression of kinasec-Src did not inhibit, but rather promoted, E4orf4 cytoplasmic death activity and phosphorylation in vivo (data not shown).

To determine whether the physical interaction between E4orf4 and kinasec-Src is required for modulation of Src-dependent phosphorylation, we analyzed the profiles of Src-dependent tyrosine phosphorylation in the presence of wild-type or mutant E4orf4 proteins. Similar levels of E4orf4 proteins were expressed together with activated c-Src (Y527F) in 293T cells, and tyrosine phosphorylation profiles were analyzed using an anti-phosphotyrosine antibody. Consistent with previous studies, the phosphorylation of a number of proteins was increased in the presence of the wild-type E4orf4, especially that of three major yet unidentified proteins of apparent molecular mass around 90, 65–70, and 45 kDa, whereas phosphorylation was not affected by expression of the nonphosphorylatable E4orf4 (4Y-F) (Fig. 5B) (13). Im-

![Fig. 4. E4orf4 binding to Src and PP2A is not competitive. A, overexpression of c-Src does not inhibit E4orf4 binding to PP2A. 293T were transfected with c-Src or PP2A alone (EV), or together with E4orf4 and immune complexes were isolated with anti-E4orf4 (2419) and analyzed by Western blot using anti-PP2A-C (pTyr) and anti-c-Src, and immune complexes were analyzed by Western blot using anti-c-Src (mab327), anti-phospho-Src (Tyr416), and anti-PP2A-C. The amounts of transfected E4orf4 and endogenous PP2A-C in total cell lysates are shown (TL). The data are representative of at least three independent experiments, and identical results were obtained using anti-FLAG to isolate FLAG-E4orf4 immune complexes.

![Fig. 5. E4orf4 binding to Src kinase domain is necessary and sufficient for modulation of Src-dependent phosphorylation. A, transfusions were performed as indicated, and immune complexes of all Src species (endogenous Src kinases alone (+) or with exogenous kinasec-Src (+)) were isolated using a pan-Src antibody (SRC2) and were analyzed using anti-phosphotyrosine (pTyr), anti-cortactin (4F11) and anti-Src (SRC2). Levels of endogenous cortactin and exogenous FLAG-E4orf4 in total cell lysates are shown (TL). Note that the level of cortactin in immune complexes containing endogenous Src and mainly kinasec-Src (Kinasec-Src) was equivalent to that in immune complexes of endogenous Src alone, in the absence of E4orf4. However, E4orf4 significantly promoted association of kinasec-Src with endogenous cortactin (asterisks) and other phosphotyrosine-containing proteins. B, equal amounts of cell lysates from 293T transfected with activated c-Src (Y527F) alone (EV) or together with wild type or the indicated mutant E4orf4 were analyzed by Western blot using RC20-HRP anti-phosphotyrosine (pTyr), anti-Src (SRC2), anti-E4orf4 (2419), or anti-β-actin. Arrows indicate major tyrosine-phosphorylated protein bands whose Src-dependent phosphorylation was increased by the wild-type and mutant E4orf4 defective in PP2A-Bo binding (R81/F84A, F84A), but not by mutant E4orf4 deficient in Src binding (6R-A) or nonphosphorylatable E4orf4 (4Y-F).]
portantly, the mutant E4orf4 (6R-A) unable to bind to kinase-\textsuperscript{Src} was also unable to modulate Src-dependent phosphorylation, similar to the nonphosphorylatable E4orf4 protein. In marked contrast, E4orf4 mutants able to interact with Src but not with PP2A-B\textsubscript{o} (R81A/F84A and F84A), still promoted the phosphorylation of specific target proteins. Thus E4orf4 binding to the kinase domain of Src family kinases likely mediates deregulation of Src signaling.

MAP kinases have been defined as essential mediators of survival and stress/death responses in several studies, generally linking the ERK1/2 pathway to survival and the stress-activated MAP kinase modules JNK and SAPK2/38 to either death or survival, depending on the context and stimuli. MAP kinases are downstream targets of Src signaling, and specific regulation by Src was reported, depending on the cell type and the nature of the stimulus (reviewed in Refs. 44 and 45). Activation of ERK downstream of Src is a major effect of the transforming viral protein polyomavirus mT that also interacts with both PP2A and Src, and contributes to trigger cell transformation and survival by mT (reviewed in Ref. 46). Notably, mT interaction with Src kinases is also mediated by the kinase domain (47). To get further biological readouts of E4orf4-Src signaling and compare the specificity of E4orf4-Src versus mT-Src signaling, we analyzed the effect of E4orf4 on the activation state of the three major MAP kinase pathways in 293T cells. Western blot analyses of cell lysates from 293T transfection with E4orf4 or mT were performed using phospho-specific antibodies that recognize the active ERK1/2, SAPK2/38, and phosphorylated c-Jun, as a measure of sustained JNK activation. None of the viral proteins had a significant effect on SAPK2/38 activity in 293T. As expected, mT led to a potent activation of ERK and also increased c-Jun phosphorylation on Ser\textsuperscript{9} in 293T cells, indicating that mT can increase the activity of two distinct non-intersecting MAP kinase pathways in the context of 293T (Fig. 6A). In marked contrast, E4orf4 did not activate ERK and a slight decrease of ERK activity was rather detected occasionally (Fig. 6A). The absence of ERK activation in E4orf4-expressing cells was consistent with the pro-death effect of E4orf4-Src signaling. Remarkably, E4orf4 expression was always associated with a robust activation of the JNK pathway leading to hyperphosphorylation of c-Jun. Mutant E4orf4 proteins that did not bind to the kinase domain of Src did not activate JNK (6R-A, 6R-A/R81A/F84A), whereas mutants defective for PP2A-B\textsubscript{o} binding (R81A/F84A, F84A) retained the capacity to activate the JNK pathway (Fig. 6B). The mutant E4orf4 (R81A/F84A) was however less efficient relative to wild-type E4orf4, suggesting that Src and PP2A signaling might cooperate in JNK activation in the context of the wild-type E4orf4. Whatever the case, deregulation of Src signaling by E4orf4 was associated with a selective JNK activation, whereas mT led to qualitatively different output signaling by activating both ERK and JNK in 293T cells.

The Cytoplasmic Death Pathway Requires Src Binding and Contributes to Cell Killing by Ad2 E4orf4—To address the requirement for Src binding for induction of the cytoplasmic death pathway typified by early changes in actin dynamics, we measured the ability of E4orf4 mutant proteins to induce early cell blebbing and chromatin condensation when expressed to similar levels in 293T cells. 293T are suitable for analyzing the cytoplasmic death activity without interference from the so-called Src-independent death activity, which is significantly delayed in this cell line (onset of the cytoplasmic death activity at 16 h post-transfection versus 60 h for the Src-independent death activity) (15). Although the cytoplasmic death activity is also manifested in other cancer cell lines (13, 15), major E4orf4 effects may be better manifested in 293T cells, because they are transformed by adenovirus E1A and E1B and thus mimic in part the cellular context in which E4orf4 normally acts. Co-transfection of a small amount of actin-GFP was used to facilitate morphological analyses in live cells (Fig. 7A, left panels). Strikingly, mutations that inhibited E4orf4 binding to the kinase domain of Src (R73A/R74A/R75A, 6R-A) completely ablated blebbing induction (Fig. 7A). In marked contrast, mutants unable to interact with PP2A-B\textsubscript{o} were only partially defective (R81A/F84A), or on the contrary, more efficient in promoting blebbing (F84A) relative to wild-type E4orf4. In a finding consistent with previous work, the ability of E4orf4 to trigger early changes in actin dynamics correlated with chromatin condensation in these cells (Fig. 6A, right panel). Thus the cytoplasmic death activity was correlated with the ability of E4orf4 to interact with kinase-\textsuperscript{Src}, to deregulate Src signaling and to activate JNK, all of which were still induced by mutants deficient in PP2A-B\textsubscript{o} binding.

Finally, clonogenic survival assays were performed to delineate the relative contributions of Src and PP2A to the overall killing activity of E4orf4. A marked lost in killing activity was observed when similar levels of the E4orf4 mutant proteins deficient in cytoplasmic death activity (E4orf4 (6R-A) and non-phosphorylatable E4orf4 (4Y-F)) were expressed in 293T cells (4-fold increase in survival), supporting a role for the Src-dependent death activity in cell killing (Fig. 7B). Nonetheless, cell killing was still induced (50%), supporting the existence of some Src-independent death activity, which is manifested later in these cells (15). Inhibition of PP2A-B\textsubscript{o} binding had less severe (R81A/F84A) or no inhibitory effect on clonogenic sur-
vival (F84A), and killing efficiencies correlated well with their respective cytoplasmic death activity in 293T cells (Fig. 7A). To compare killing efficiencies in a background where PP2A-Bα binding and Src-independent killing were reported to play more prominent roles, we performed similar morphological and clonogenic assays in the human cervical carcinoma line C-33A (15, 18). In these cells, expression of a wild-type E4orf4-GFP protein led to the appearance of two different morphological phenotypes (also induced by untagged E4orf4). Some cells were presenting prominent cytoplasm blebs characteristic of the cytoplasmic death activity (Fig. 8A, arrowhead 1), but a large proportion were showing dramatic cell shrinkage and rounding in the absence of blebbing (Fig. 8A, arrowhead 2). In marked contrast, no typical blebbing cells were observed upon expression of E4orf4 (6R-A) or (4Y-F), which are both deficient in cytoplasmic death activity. However these mutant proteins still induced the distinct morphological phenotype associated with Src-independent killing (Fig. 8A, arrowhead 2). The E4orf4 (6R-A) mutant that is partially defective in PP2A-Bα binding (Fig. 3B) was also less efficient than the nonphosphorylatable E4orf4 in induction of the Src-independent phenotype. Nevertheless, significant killing was induced by this mutant protein, whereas the mutant unable to bind to PP2A-Bα (R81A/F84A) was much more defective in this particular cell background.

Fig. 7. Inhibition of Src binding, but not PP2A-Bα binding, abolishes the cytoplasmic death activity of Ad2 E4orf4. A, the cytoplasmic death activity was analyzed in 293T cells during the first 48 h after transfection. Blebbing was monitored by cotransfecting actin-GFP with E4orf4 constructs at a plasmid DNA ratio of 1:20, and the amounts of GFP-positive blebbing cells were determined by fluorescence microscopy in live cells 24 h after transfection (left panel). Data are expressed as percent blebbing cells relative to the total number of GFP-positive cells (right graph, light gray bars). Other cultures transfected with E4orf4 proteins only were fixed 48 h after transfection and processed for immunostaining of E4orf4 proteins and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) labeling. Nuclear condensation was determined by counting the number of E4orf4-positive cells presenting nuclear shrinkage associated with intense chromatin condensation, and data are expressed relative to the total number of E4orf4-positive cells (dark gray bars). Aliquots of transfected cells were kept for Western blot analysis of expression levels (right inset). Data are the mean ± S.D. of at least three independent experiments, n > 1,000, and identical results were obtained using N-terminal-tagged or -untagged E4orf4 proteins, as well as E4orf4-GFP fusion proteins. Bars, 10 μm. B, 293T were transfected with pGKpuro together with either the vector only (EV), wild-type, or mutant E4orf4, as indicated, using a plasmid DNA ratio of 1:20, and cell survival assays were performed. Aliquots of transfected cells were kept for Western blot analyses of expression levels 24 h after transfection, before applying the selection for transfected cells (puromycin, 3 μg/ml). Percentages of surviving cells were obtained by counting the number of resulting colonies and are expressed relative to the total number of colonies obtained in cells transfected with the vector only (EV). Data are representative of at least three independent experiments (means ± S.D.), and identical results were obtained with the N-terminal-tagged and -untagged E4orf4.
binding in cell killing. Our findings support the existence of at least two major and distinct death pathways, which both contribute to cell killing by E4orf4 within a given cell population, but whose relative contributions vary in function of the cellular background. Evidence was obtained that one death pathway requires Src binding and may involve some cooperative signaling between Src and PP2A, whereas the second rather relies on the ability of E4orf4 to bind to PP2A-Ba.

**Binding to the Kinase Domain of Src as a Dominant Mechanism to Deregulate Src Family Kinase Activity**—To clarify the mechanisms by which E4orf4 deregulates Src tyrosine kinase signaling and how killing depends on Src, we have delineated the molecular determinants of a physical interaction between c-Src and E4orf4, showing that the Arg-rich motif of E4orf4 binds to the kinase domain of c-Src. Furthermore, we have provided convincing evidence that this physical interaction mediates E4orf4 deregulation of Src-dependent signaling and is required for triggering the cytoplasmic death program. Indeed, mutation of this Arg-rich motif completely ablated E4orf4-dependent modulation of Src phosphorylation, JNK activation, and the morphological changes that typify the cytoplasmic death pathway, without completely inhibiting E4orf4 functions (residual binding to PP2A-Ba and killing). Thus the possibility that this loss of function in cytoplasmic death activity may result from major misfolding problems is rather unlikely. The solubility of all the mutant E4orf4 proteins was similar to that of wild-type E4orf4, and immunofluorescence analyses confirmed that the proteins were not aggregated (data not shown). Based on these results, we propose that direct binding to the kinase domain of Src is the critical and rate-limiting step for activation of E4orf4 cytoplasmic death function. Src binding to the Arg-rich motif may allow the stable accumulation of E4orf4 in the cytoplasm and membranes. Indeed, E4orf4 preferentially accumulates in the nucleus when expressed in limited amounts (13), and the Arg-rich motif and surrounding sequences regulate its nuclear accumulation.² Binding to the Src kinase domain may mask some critical sequences required for nuclear import and promotes E4orf4 phosphorylation, a process that further stimulates its association with the membrane-actin skeleton (13). These results indicated that the kinase domain of Src can support the formation of at least some E4orf4-Src signaling complexes (binding to phosphotyrosine proteins and cortactin), as well as E4orf4 phosphorylation in vivo (data not shown). Thus the physical interaction may activate Src and subsequently the phosphorylation-dependent cytoplasmic activity of E4orf4. This is consistent with our previous findings suggesting that endogenous Src proteins associated with E4orf4 are activated (Tyr⁴¹⁶ phosphorylation), but that E4orf4 association with c-Src does not require E4orf4 tyrosine phosphorylation and Src catalytic activity (13). Most interactions with Src kinases occur through the SH2 and SH3 domains and disrupt the intramolecular interactions that maintain the kinases in a so-called inactive conformation (reviewed in Ref. 48). Few cellular ligands for Src kinase domain were reported, including the adaptor β-arrestin 1 (49), the trimeric Go proteins (50), and the Shc adaptor protein (51), but little is known regarding the physiological functions of such unusual protein-protein interactions taking place within the catalytic domain. However, there is evidence that they can activate the tyrosine kinase (52). We observed a modest 2-fold increase of Src activity (Tyr⁴¹⁶ phosphorylation) in E4orf4-expressing cell lysates relative to control cell lysates (data not shown). Considering that a limited fraction of E4orf4 is associated with cellular Src, this may well be significant. Thus available evidence strongly suggests that E4orf4 act by promoting specific Src signaling via binding to the kinase domain. Recruitment of active Src sig-

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**DISCUSSION**

This study addresses a critical question in cell biology related to the mechanisms by which adenoviral proteins usurp the function of cellular proteins to control the death machinery. The emerging complexity of E4orf4 killing functions in different systems (14–18) has raised the need for systematic analyses that consider E4orf4 activities in relation with potential targets and overall killing within the same cellular background, as compared with other cell types. Such an approach is critical to better comprehend the tumor-selective activity of E4orf4 in human transformed cells. Here we provide two original and critical findings that clarify some issues regarding E4orf4 killing in transformed cells. The first is related to the mechanism developed by E4orf4 to deregulate Src tyrosine kinase signaling by physically binding to the kinase domain. The second regards the differential roles of Src and PP2A-Ba

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**Fig. 8.** Cell death activities of wild-type and mutant E4orf4 proteins in C-33A cells. A, C-33A cells were transfected with the indicated E4orf4-GFP constructs and the morphological phenotypes of E4orf4-positive cells were analyzed by fluorescence microscopy 24 h after transfection. Arrow numbers indicate the two major cell death phenotypes observed for each E4orf4 protein: arrowhead 1, blebbing phenotype characteristic of the cytoplasmic death activity; arrowhead 2, distinct regular cell rounding and shrinkage typical of the Src-independent death activity. The broad spectrum caspase inhibitor Boc-Asp-(OMe)-CH₂F (BocD-fmk) was added to the cultures during and after transfection.

B, Arrowhead 2. Survival (% cells). Residual killing by this mutant was more often associated with the Src-dependent phenotype (Fig. 8A, arrowhead 1). Thus both E4orf4 binding to Src and PP2A appear to play critical roles in cell killing, and their contributions may vary in the function of the cell background.
naling complexes at specific membrane sites may deregulate critical Src functions in transformed cells, including actin dynamics (12). The first described ligand for Src kinase domain was another DNA virus protein, the transforming polyomavirus middle T-antigen (47). Similarly, polyomavirus middle T binding to the Src kinase domain leads to its tyrosine phosphorylation and subsequent recruitment and phosphorylation of cellular proteins (reviewed in Ref. 46). However, the final outcome is completely different, middle T transforms normal cells, whereas Ad2 E4orf4 kills transformed cells. The different outcomes are likely dictated by the nature of the signaling proteins being recruited to phosphorylated middle T versus phosphorylated Ad2 E4orf4. Indeed, mT does not tend to bind proteins involved in down modulation of signaling (e.g. GAP proteins), whereas E4orf4 does. Our data also indicated that mT and E4orf4 differentially modulate the activity of the MAP kinase pathways ERK and JNK. Indeed, mT potently activates ERK, whereas E4orf4 does not, rather triggering a sustained activation of the JNK pathway in a Src-dependent manner. We have already shown that E4orf4 leads to inhibition of Src-Fak signaling and disorganization of focal adhesion complexes (12). This could be sufficient to block a cell survival signal linked to the regulation of cell shape, as Fak promotes survival through the activation of various signaling pathways, including ERK and PKB (53, 54). Whether the imbalance in MAP kinase signaling induced by E4orf4 contributes to cell death induction, or is only a consequence of E4orf4 ability to redirect Src signaling to specific targets/compartments and deregulate actin dynamics remains to be determined in future studies. Whatever the case, viral proteins have developed mechanisms to deregulate normal cellular functions in a way that cannot be restricted by other cellular factors. The observation that two viral proteins utilize a similar molecular mechanism to usurp Src functions is likely of physiological relevance. It could be that such a mode of Src regulation by other cellular factors plays a role during pathological cell proliferation and death processes. Despite intensive studies, Src functions in tumorigenesis and programmed cell death are still not clearly understood, and mT and E4orf4 will certainly provide powerful molecular tools for furthering our knowledge. It was reported that two basic motifs separated by negative charges, each followed by a serine or a threonine, are essential for middle T interaction with Src kinases (PKRRSEELRRAAAT) (21). This motif is strikingly similar to the minimal sequence on E4orf4 that mediates binding to Src kinase domain in vitro (62-YYTERR-AKRDRRRRSVCH-79). It will be interesting to determine whether other cellular ligands also use similar basic motifs and what sequences within Src kinase domain are involved.

Src and PP2A Signaling May Specify the Nature of E4orf4 Cell Death Activity in the Function of the Cellular Background—To identify the sequences that mediate binding to Src kinase domain and to delineate the contribution of Src to the cytoplasmic death pathway relative to overall cell killing, we have used E4orf4 mutant proteins characterized for their in vivo association with PP2A-Ba (18). In previous work, their killing activity was assessed by long term colony-forming assays. Here, we have considered more proximal effects, notably deregulation of Src phosphorylation, MAP kinase signaling, and induction of the dramatic blebbing phenotype. We believe that the results obtained add several findings of critical importance for interpretation of loss-of-function regarding E4orf4 mutants. In vitro binding assays revealed that mutation of the Arg-rich motif directly inhibits the molecular association with c-Src kinase domain, regardless of its putative effect on E4orf4 localization. Such mutations also affected, although to a lesser extent, the in vivo association with PP2A-Ba. Whether mutation of the Arg-rich motif affects PP2A-Ba binding at the molecular level, or if decreased Src binding and/or alteration of E4orf4 localization may account for decreased PP2A association in vivo is unclear. Given the lack of knowledge regarding the molecular determinants of the physical E4orf4-PP2A-Ba interaction in vitro and specific targets of E4orf4-PP2A-Ba in mammalian cells, we are unable yet to appreciate the real contribution of PP2A-Ba binding to the cytoplasmic death activity in the context of the wild-type E4orf4. Indeed, interactions between Src, PP2A, and E4orf4 are likely to be complexes in vivo, given that Src and PP2A can also interact with one another (96–40). This raises the possibility that E4orf4 may still associate with PP2A in the absence of direct binding to PP2A-Ba, in a Src-dependent manner. Additionally, Src can phosphorylate the catalytic subunit of PP2A and inhibit the phosphatase activity (37, 38, 40, 55). Because some E4orf4 complexes can contain both proteins, PP2A-C could well be a target of E4orf4-Src signaling. We have been unable to measure an increase in PP2A-C tyrosine phosphorylation in E4orf4-expressing cells (data not shown), but rapid autodephosphorylation in vitro may have hindered detection (55). Whatever the case, our data indicated that Src and PP2A associations are not mutually exclusive and suggested that they can cooperate to induce some of the specific E4orf4 activities and efficient cell killing in the context of wild-type E4orf4. Transient deactivation of PP2A by E4orf4-Src could enhance transmission of E4orf4-Src signals locally. Indeed, simultaneous binding to Src and PP2A could potentiate JNK activation and hyperphosphorylation of c-Jun by E4orf4, as suggested by the decrease in c-Jun hyperphosphorylation in cells expressing the R81A/F84A mutant relative to those expressing the wild-type E4orf4. Clarification of the exact nature of this cooperative signaling will require a better comprehension of the direct effects of E4orf4 on the phosphatase activity of PP2A.

Despite the complexity of E4orf4, Src, and PP2A signaling, we have succeeded in partially discriminating the functional roles of Src and PP2A-Ba binding in the cytoplasmic death program. This was achieved by using E4orf4 mutants still able to support efficient Src binding but completely defective for PP2A-Ba binding (R81A/F84A, F84A). Functional assays revealed that although PP2A signaling may contribute, E4orf4 binding to PP2A-Ba is not required to engage the cytoplasmic death pathway, which rather depends on the ability of E4orf4 to interact with the kinase domain of Src. Furthermore, the E4orf4 (F84A) mutant repeatedly showed a gain-of-function in cytoplasmic death activity, which correlated with a 2-fold increase in killing in 293T cells. Because binding to PP2A-Ba and Src does not appear to be competitive and binding to Src was not promoted, this mutation may rather increase E4orf4 association with and/or regulation of some other major targets in the cytoplasmic death pathway. Our preliminary work suggests that this gain-of-function is related to the ability of E4orf4 to modulate the Rho GTPases and actin dynamics. Nonetheless, because residual killing remained upon inhibiting Src binding (this study) and Src tyrosine kinase activity (12, 15), a distinct cell death pathway independent of E4orf4 tyrosine phosphorylation exists, which appears to be critically dependent on PP2A-Ba binding, as reported previously (10, 18, 56). This distinct cell death pathway was more efficient in C-33A cells and was characterized by a distinct cell phenotype (cell shrinkage and lifting without membrane blebbing). Additionally, early cell death induced by E4orf4 (6R-A) and non-phosphorylatable E4orf4 in C-33A was not markedly inhibited by calpain inhibitors, whereas coexpression of calpastatin significantly inhibited the cytoplasmic cell death activity mani-
fested by the wild-type and E4orf4 (RS1A/FS4A) mutant (data not shown), adding support for distinct death pathways, as previously reported (15). The loss of function manifested by E4orf4 (RS1A/FS4A), and the decreased efficiency of E4orf4 (6R-A) that correlated with its partial defect in P2PA-Ba binding, support a role for P2PA-Ba binding in engagement of this distinct cell death pathway. Importantly, both cell death activity contributed to overall killing in two different cell lines, although to a different extent: the cytoplasmic death activity was higher in 293T cells, whereas the phosphorylation-independent death activity was more predominant in C-33A cells. Although the existence of two distinct cell death pathways was suggested using a molecular targeting approach, this work provides the first evidence that they are manifested in the context of normal E4orf4 signaling within a given cell population. We believe that this work will provide critical information for future studies aimed at deciphering the specific effects of E4orf4 in normal versus transformed cells.

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Activation of Adenovirus Type 2 Early Region 4 ORF4 Cytoplasmic Death Function by Direct Binding to Src Kinase Domain
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