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Inhibition of the Raf-1 Kinase by Cyclic AMP Agonists Causes Apoptosis of v-abl-Transformed Cells

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Here we investigate the role of the Raf-1 kinase in transformation by the v-*abl* oncogene. Raf-1 can activate a transforming signalling cascade comprising the consecutive activation of Mek and extracellular-signal-regulated kinases (Erks). In v-abl-transformed cells the endogenous Raf-1 protein was phosphorylated on tyrosine and displayed high constitutive kinase activity. The activities of the Erks were constitutively elevated in both v-raf- and v-abl-transformed cells. In both cell types the activities of Raf-1 and v-raf were almost completely suppressed after activation of the cyclic AMP-dependent kinase (protein kinase A [PKA]), whereas the v-abl kinase was not affected. Raf inhibition substantially diminished the activities of Erks in v-raf-transformed cells but not in v-abl-transformed cells, indicating that v-abl can activate Erks by a Raf-1-independent pathway. PKA activation induced apoptosis in v-abl-transformed cells while reverting v-raf transformation without severe cytopathic effects. Overexpression of Raf-1 in v-abl-transformed cells partially protected the cells from apoptosis induced by PKA activation. In contrast to PKA activators, a Mek inhibitor did not induce apoptosis. The diverse biological responses correlated with the status of c-*myc* gene expression. v-abl-transformed cells featured high constitutive levels of expression of c-*myc*, which were not reduced following PKA activation. Myc activation has been previously shown to be essential for transformation by oncogenic Abl proteins. Using estrogen-regulated c-*myc* and temperature-sensitive Raf-1 mutants, we found that Raf-1 activation could protect cells from c-*myc*-induced apoptosis. In conclusion, these results suggest (i) that Raf-1 participates in v-abl transformation via an Erk-independent pathway by providing a survival signal which complements c-*myc* in transformation, and (ii) that cAMP agonists might become useful for the treatment of malignancies where *abl* oncogenes are involved, such as chronic myeloid leukemias.

Raf-1, the cellular homolog of the v-raf oncoprotein, is a ubiquitously expressed serine/threonine kinase which serves as a central interface in the transmission of mitogenic signals from the cell membrane to the nucleus. Raf-1 is activated by various growth factors, and its function has been shown to be required for transformation by several classes of oncogenes, including ligands, tyrosine kinase receptors, Ras proteins, and src family tyrosine kinases (recently reviewed in references 18, 50, and 98). Recent investigations have charted a pathway indicating how Raf-1 links membrane-bound signalling molecules to nuclear transcription factors. Upon activation, many growth factor receptors associate with adaptor proteins, such as grb-2, crk, and shc, which in turn recruit guanine nucleotide-releasing proteins (GNRP), such as SOS and C3G, to the plasma membrane. GNRP activate Ras proteins by mediating the exchange of GDP to GTP (29, 62; for reviews, see references 63 and 68). GTP-loaded Ras proteins can bind to the N-terminal region of Raf-1 with high affinity (reviewed in reference 68), causing the translocation of Raf-1 from the cytosol to the membrane, where Raf-1 is exposed to activators (58, 89). The nature of the physiological Raf-1 activator(s) is not yet clear but might include protein kinase C (6, 52, 87) and src family tyrosine kinases (26, 60, 94, 99). Activated Raf-1 in turn can phosphorylate Mek, a dual-specificity kinase which activates the extracellular signal-regulated kinases Erk-1 and -2 by phosphorylation on tyrosine and threonine residues (for reviews, e.g., see references 18, 61, and 98). Activated Erks can translocate to the nucleus and phosphorylate transcription factors. The best-studied nuclear target of Erks is the ternary complex factor, which is required for induction of the c-*fos* gene (34, 54). The c-Fos protein associates with c-Jun to form the AP-1 transcription factor (reviewed in reference 16), which is a main mediator of v-raf transformation (53, 54, 73). The activation of this signalling cascade leading from the cell membrane to the nucleus seems to be both necessary and sufficient for transformation of NIH 3T3 fibroblasts (14).

A number of recent reports have identified the cyclic AMP (cAMP)-dependent kinase protein kinase A (PKA) as a negative regulator of this pathway. Activation of PKA blocks the activation of Erks in several different cell types, including fibroblasts and smooth muscle cells (4, 11, 36, 38, 40, 80, 91, 101). PKA does not affect the activity of Ras, Mek, or mitogen-activated protein kinase (MAPK) (11, 101) but was shown to both inhibit Ras-dependent activation of Raf-1 (4, 11, 101) and downregulate Raf-1 kinase activity directly (38). Phosphorylation of the Raf-1 regulatory domain by PKA decreases the affinity of Raf-1 for activated GTP-loaded Ras, thereby preventing the activation of Raf-1 (38, 101). In addition, phosphorylation of the kinase domain directly suppresses the catalytic activity of the Raf-1 kinase domain (38). This type of inhibition is dominant, as both activated Raf-1 and the dereg-

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ulated Raf-1 kinase domain are susceptible to PKA inhibition. As a consequence, PKA is able to abolish the anchorage-independent growth of NIH 3T3 fibroblasts in soft agar (38).

In this study we have investigated the role of Raf-1 in transformation by the v-*abl* tyrosine kinase oncogene. c-*abl*, the cellular homolog of v-*abl*, is an important target of mutations implicated in the genesis of human tumors, not*abl*y in chronic myeloid leukemia and acute lymphocytic leukemia (reviewed in references 56, 79, and 92). In these diseases *abl* is activated due to chromosomal translocations which result in the expression of Bcr-Abl fusion proteins that, like v-abl, display unrestrained tyrosine kinase activity. Abl proteins can couple to several different signalling pathways which contribute to oncogenic transformation. v-abl associates with and activates the phosphatidylinositol 3-kinase (96), which is also found in complexes with c-abl and Bcr-Abl proteins (81). Furthermore, Abl proteins bind to crk (28, 74), shc (69), and grb-2 (76) adaptor proteins, which are involved in Ras activation. Oncogenic Abl proteins also induce expression of c-*myc* by a yet unknown mechanism (2, 8, 75, 100). Both the deregulation of c-*myc* expression and the activation of Ras have been shown to be required for transformation by *abl* oncogenes (44, 95).

While the pathways mediating Abl-induced expression of c-*myc* remain enigmatic, Ras proteins can signal through a number of different effector molecules, including Raf-1 (reviewed, for example, in reference 27). The strong synergism between v-*raf* and v-*myc* oncogenes in terms of transformation (9, 72; for a review, see reference 51) led us to assess the effects of Raf-1 inhibition on v-abl transformation. Dominant negative Raf-1 mutants associate with the Ras effector domain stably enough to prevent other proteins, such as phosphatidylinositol 3-kinase, GTPase-activating protein, neurofibromin, and B-raf, from binding to this domain (67), suggesting that dominant negative Raf-1 mutants inhibit Ras signalling in general. Therefore, we utilized PKA activators as tools to inhibit Raf-1 independently of Ras. The kinase activity of Raf-1 was massively elevated in v-abl-transformed cells, presumably due to tyrosine phosphorylation. PKA activation completely inhibited Raf-1 activity in v-abl-transformed cells and precipitated apoptosis. In contrast, PKA activation induced reversion of the transformed phenotype in v-raf-transformed cells. A hallmark of v-abl-transformed cells is the constitutive expression of c-*myc*, which is not affected by PKA activation. As c-*myc* is known to induce apoptosis in growth factor-deprived cells (reviewed in reference 23), we tested whether selective Raf-1 activation could prevent c-*myc*-triggered apoptosis. Using conditional *raf*-1 and c-*myc* alleles, we could show that Raf-1 activation indeed abrogated c-*myc*-induced apoptosis. Thus, our results suggest that Raf-1 contributes to v-abl transformation by providing a survival function which complements c-*myc*.

MATERIALS AND METHODS

Cells and reagents. Cells were grown in Dulbecco modified Eagle medium (DMEM; Serva) supplemented with glutamine and 10% fetal calf serum (FCS; Seromed). Cells were made quiescent by a 24-h incubation in DMEM without serum. Transformed cells were generated by transfection of NIH 3T3 cells with expression vectors encoding the v-*raf* (38) or the v-*abl* (57) oncogene or a Raf-1 expression vector as described previously (39). To exclude clonal variation, pools of transformed cells were used. 8-chloro-cAMP (8-Cl-cAMP), 8-bromo-cAMP, CTP-cAMP, dibuturyl-cAMP, 5,6-dichlorobenzimidazole riboside-3′,5′-monophosphorothioate (Sp isomer) (Sp 5,6-DCl-c-BIMPS), and forskolin were purchased from Sigma, ICN, or Biolog. The specific Mek inhibitor PD98059 (19) was purchased from New England Biolabs. The Raf antiserum (crafVI) was raised in chinchilla bastard rabbits immunized with a synthetic peptide corresponding to the 12 carboxy-terminal amino acids of Raf-1. It recognizes both Raf-1 and v-raf and was used at a 1:750 dilution for immunoprecipitation and at a 1:2,000 dilution for Western blotting. The B-Raf antiserum was described previously (41). It was raised in chinchilla bastard rabbits immunized with a synthetic peptide corresponding to the 12 carboxy-terminal amino acids of B-Raf. The MAPK antibody was kindly provided by Peter Shaw. This antiserum was raised by immunizing chinchilla bastard rabbits with purified Erk-2. It was used at a 1:250 dilution for immunoprecipitation. For Western blotting a monoclonal mouse anti-Erk antibody (Transduction Laboratories) was used. The v-abl monoclonal antibody was from Oncogene Science, and the antiphosphotyrosine antibody (PY-20) was from UBI.

Growth curves and soft agar assays. For growth curves, duplicates of 10^4 cells per well were seeded in a 24-well dish in the presence of 10% FCS. In one set, 100 μM 8-Cl-cAMP or 50 μM PD98059 was added as a single dose 6 h after plating. Cells were harvested at the time points indicated in the figures, and the number of viable cells was counted. For colony growth in soft agar, 10^4 cells were resuspended in 3 ml of 0.4% soft agar (Noble agar; Difco) containing DMEM plus 10% FCS and plated in six-well plates. The agar was overlaid with medium to achieve a final concentration of 100 μM 8-Cl-cAMP. The overlay was replenished every 3 days. Pictures were taken on day 10.

Construction of cells expressing conditional *raf*-1 and c-*myc* genes. These cell lines were derived from the TGR-1 cell line (71), which is a subclone of the Rat-1 cell line. Temperature-sensitive mutants of Raf-1 kinase were constructed by site-directed mutagenesis of the isolated kinase domain cloned in a retrovirus vector (39) and will be described in detail elsewhere. The mutant used in this work, R391/E393, has the arginine residue at position 391 and the glutamic acid residue at position 393 of full-length human c-Raf-1 changed to alanines. TGR-Raf^{ts}-E5M is a clonal cell line derived from TGR-1 by infection with the EC12-R391/E393 retrovirus and selection with G418. TGR-Raf^{ts}-E5M cells display a transformed morphology, form foci, and grow in soft agar at 33°C (the permissive temperature). They display a normal morphology and fail to form foci or grow in soft agar at 39.5°C (the nonpermissive temperature). TGR-Raf^{ts}-MycER-1 is a clonal cell line derived from TGR-Raf^{ts}-E5M by infection with a retrovirus vector, LXSH (64), expressing a c-Myc–estrogen receptor (Myc-ER) chimeric protein (21) and selection with hygromycin. Thus, TGR-Raf^{ts}-E5M cells express a Raf kinase domain whose activity is regulatable with temperature and TGR-Raf^{ts}-MycER-1 cells express the temperature-sensitive Raf kinase plus a c-Myc protein whose activity is regulatable with β-estradiol. In TGR-Raf^{ts}-MycER-1 cells, both Raf and Myc are conditional and can be regulated independently: at 39.5°C without β-estradiol, Raf is off and Myc is off; at 39.5°C with β-estradiol, Raf is off and Myc is on; at 33°C without β-estradiol, Raf is on and Myc is off; and at 33°C with β-estradiol, Raf is on and Myc is on. Cell lines were passaged in DMEM containing 10% FCS in the absence of β-estradiol at 39.5°C.

Apoptosis assays. To assay apoptosis in TGR-1 cells and derivatives, cells were plated into six-well dishes. Cells were seeded such that at the point of serum deprivation, all wells were at the same degree of confluence (approximately 70%). The time between seeding and serum deprivation was 24 to 48 h, and during this interval cells were grown at 39.5°C in the absence of β-estradiol. Temperature shift-down and/or addition of β-estradiol was coincident with serum deprivation. Nonadherent cells were collected at 12-, 18-, and 24-h time points, as indicated above, and counted in a Coulter counter. Remaining adherent cells in each well were trypsinized and likewise counted. Apoptosis values presented in Fig. 5 are ratios of floating cells to total cells, expressed as percentages. The detached cells represent apoptotic cells, as was confirmed by testing for endonucleosomal DNA fragmentation by electrophoresis of genomic DNA and in situ labeling with terminal transferase (data not shown). All treatments in a single experiment were done at the same time; the entire experiment was performed on three separate occasions with consistent results. A representative experiment is shown. The 12- and 24-h time points gave values consistent with the 18-h values, which are shown in Fig. 5A. Apoptosis was further assayed by a terminal deoxynucleotidyl transferase-mediated dUTP nicked-end labeling assay. Briefly, floating cells were collected in a 15-ml tube, and attached cells were washed with 1 ml of phosphate-buffered saline (PBS), trypsinized in 1 ml of trypsin, and collected in the same tube. Cells were spun down, resuspended in 3 ml of PBS, and aliquoted into two poly-L-lysine (Sigma)-coated Nunc chamber slides. Slides were centrifuged at 1,000 rpm for 5 min, and cells were fixed with 100% methanol (10 min at room temperature), rehydrated with PBS, and stored at 4°C. For the fragment elongation reaction, the slides were incubated in 25 μl of reaction mix (5 μl of 5× TdT buffer and 0.5 μl of TdT [BRL], 0.5 μl of 1 mM digoxigenin 11-dUTP, 1 μl of 0.5 mM dTTP, 18 μl of double-distilled water) in a moist chamber for 2 h at 37°C. Subsequently, the slides were washed three times with PBS and incubated with a rhodamine-conjugated antidigoxigenin antibody (20 μg/ml in 4× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 10 mg of BSA per ml, 0.1% Tween 20) for 45 min. After being washed three times with 4× SSC containing 0.1% Tween 20, the cells were mounted and examined in a fluorescence microscope.

Apoptosis of normal and v-raf- or v-abl-transformed NIH 3T3 cells was examined by a flow-cytometric method as described previously (13, 22) or by DAPI (4′,6-diamidino-2-phenylindole) staining. For flow cytometry, cells were treated with drugs as indicated for Fig. 3A, harvested, and washed once in PBS containing 10% FCS. Necrotic cells were identified by staining them with 0.02 μg of propidium iodide (Sigma) per ml. Propidium iodide-negative cells were examined for forward and side scatter with a FACScan flow cytometer and the Cell Quest analysis program (Becton Dickinson). DAPI staining was performed on cells grown on coverslips as follows: cells were fixed with methanol-acetone (1:1) for 2 min. Subsequently, the slides were washed once with PBS and stained with DAPI (0.5 g/ml; Sigma) dissolved in 20% glycerol-PBS. After removal of the

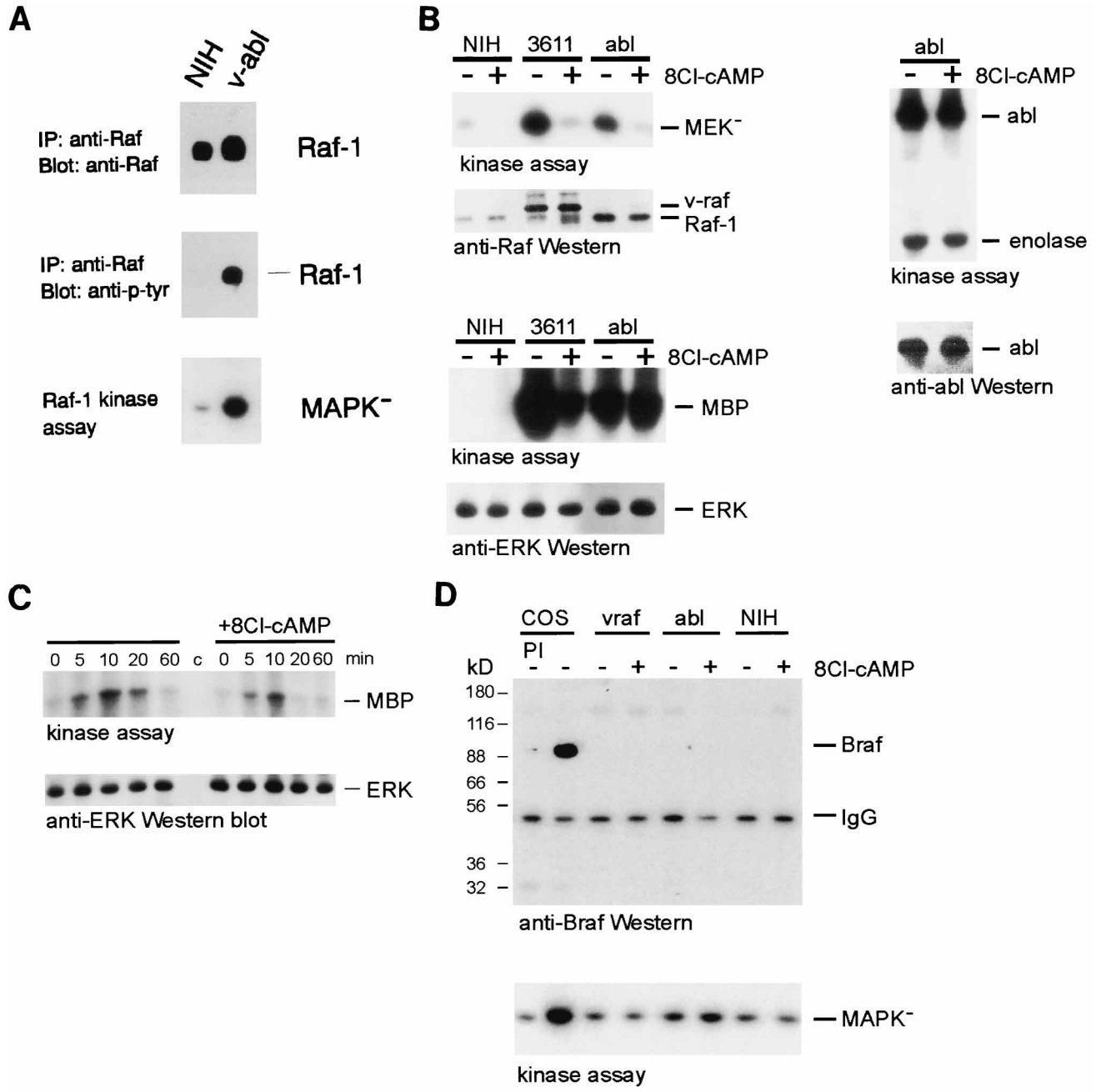
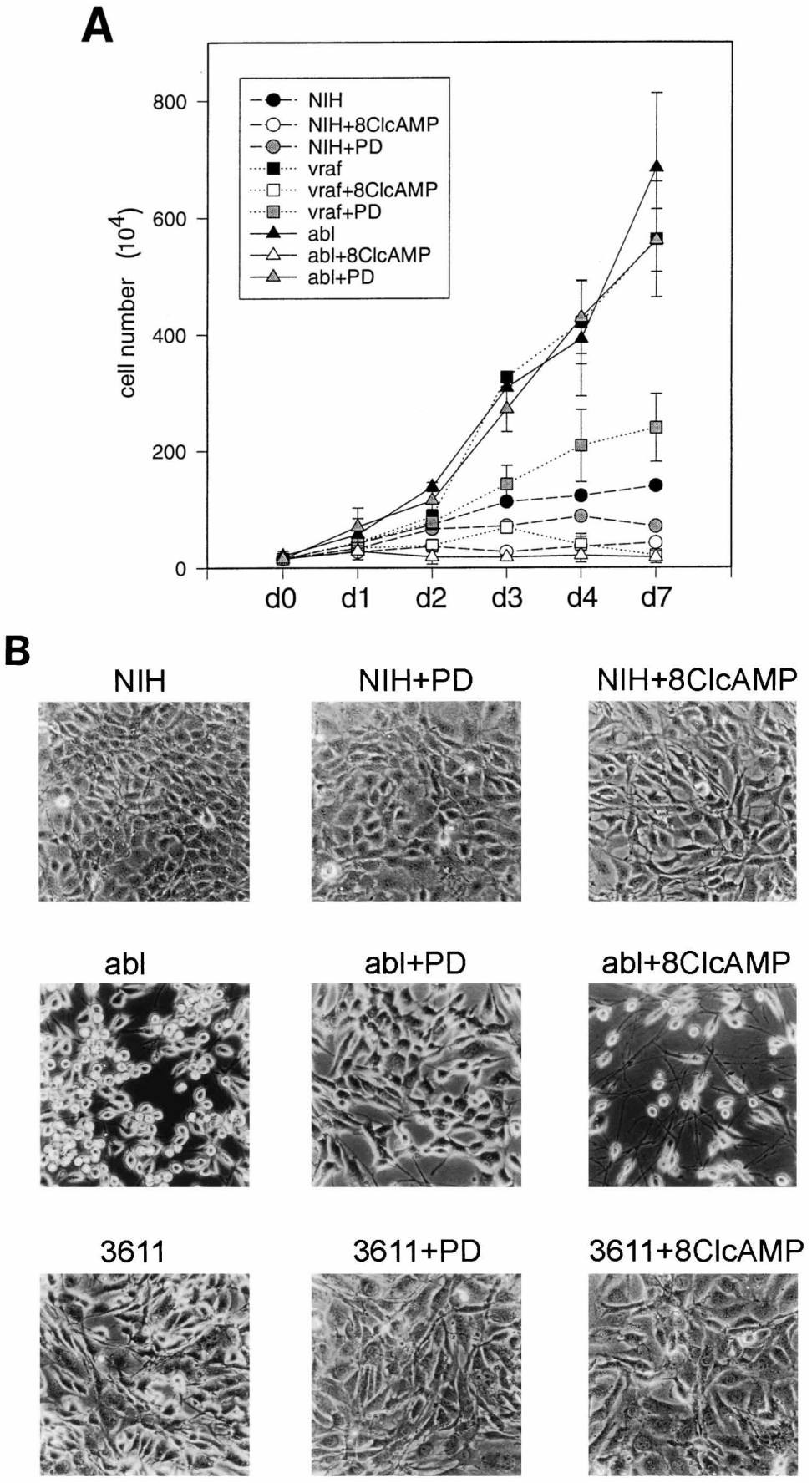


FIG. 1. Influence of 8-Cl-cAMP on the activity of Raf and MAPK. (A) Raf-1 is tyrosine phosphorylated and constitutively activated in v-abl-transformed cells. Raf-1 was immunoprecipitated from growing NIH 3T3 and v-abl-transformed cells and examined for kinase activity by a linked-kinase assay. This assay tests the ability of Raf to activate recombinant Mek-1, whose catalytic activity is measured with kinase-negative Erk protein ($MAPK^-$) as the substrate (33). The kinase reaction products were separated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The blot was autoradiographed and sequentially stained with antiphosphotyrosine (anti-p-tyr) and anti-Raf antibodies. (B) PKA activation inhibits activated Raf-1 and v-raf but not the v-abl tyrosine kinase. Growing cells were treated with 100 μM 8-Cl-cAMP for 30 min as indicated (+). The lysates were immunoprecipitated with antisera against Erk-1, Erk-2, v-abl, and Raf proteins. The Raf antiserum used recognizes both Raf-1 and v-raf. The activities of the different kinases were examined with the following substrates: kinase-negative Mek-1 (MEK^-) for Raf-1 and v-raf, myelin basic protein (MBP) for Erks, and enolase for v-abl. The kinase reaction products were separated by SDS-polyacrylamide gel electrophoresis, subsequently blotted, and examined for the amount of kinase present by Western blot analysis. 8-Cl-cAMP did not affect the kinase activity of v-abl, whereas it almost abolished the activities of both Raf-1 and v-raf. As a consequence of v-raf inhibition, the activity of Erks was substantially reduced in v-raf-transformed cells. In contrast, the constitutive Erk activity in v-abl-transformed cells was not suppressed. (C) Time course of serum-induced Erk activation in v-abl-transformed cells. Serum-starved cells were treated with 100 μM 8-Cl-cAMP where indicated for 30 min and subsequently stimulated with 20% FCS for 5, 10, 20, or 60 min. Erks were immunoprecipitated and assayed for kinase activity with myelin basis protein. The kinase reaction products were run on an SDS-polyacrylamide gel, subsequently blotted, and examined for equal amounts of Erk in the samples by Western blot analysis. Lane c is a control where Erk immunoprecipitates were omitted. To highlight the dampening of Erk activation by 8-Cl-cAMP after serum induction, a very short exposure of the gel is shown. (D) Western blot and kinase assays of B-Raf. Cell lysates were immunoprecipitated with a B-Raf antiserum, and kinase activity was examined with Mek-1 and kinase-negative MAPK ($MAPK^-$). The samples were subsequently run on an SDS-polyacrylamide gel, blotted, and examined for the presence of B-Raf with the B-Raf antiserum. COS cells transfected with a B-Raf expression vector served as a positive control. As a negative control, lysates from B-raf-transfected COS cells were immunoprecipitated with the corresponding preimmune serum (PI). Braf, B-Raf; vraf, v-raf; IgG, immunoglobulin G.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vraf
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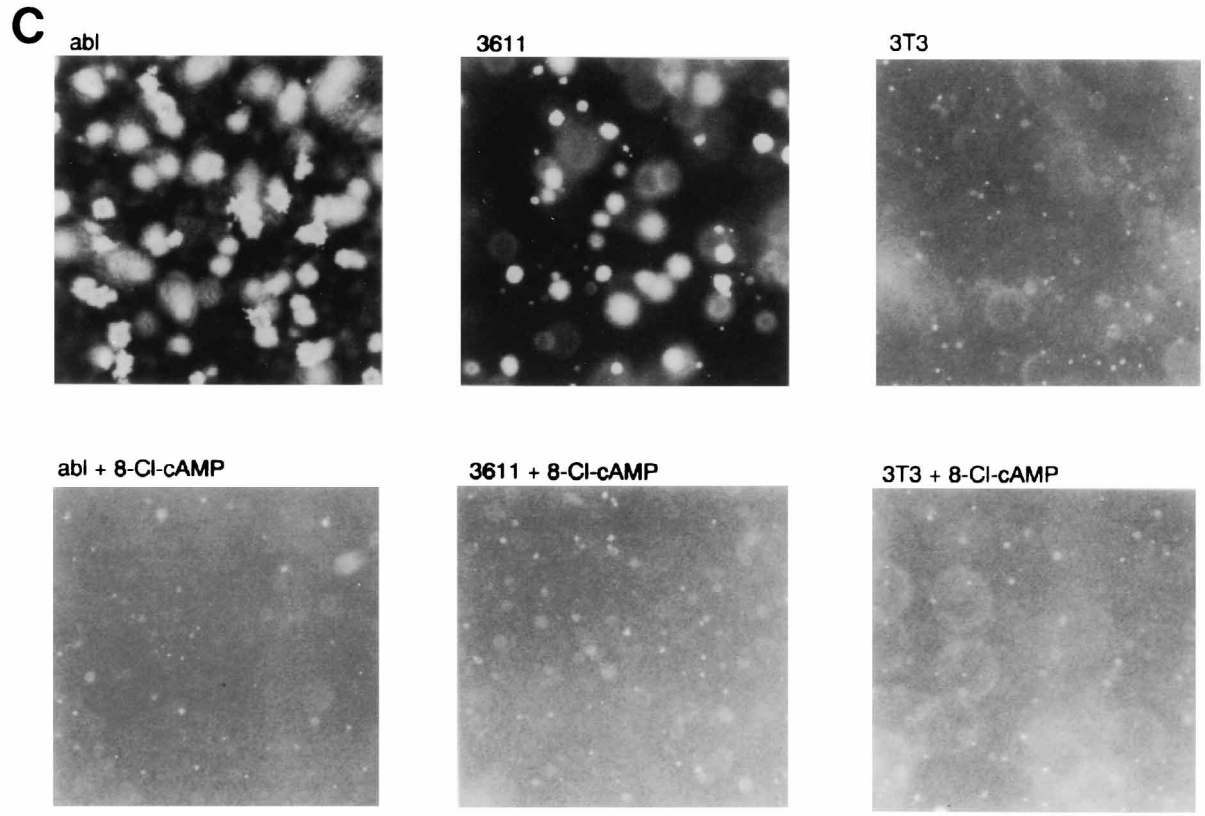


FIG. 2. PKA activation antagonizes transformation. (A) Growth curves of v-abl-transformed v-raf-transformed (3611), and parental NIH 3T3 cells treated with 8-Cl-cAMP. Equal numbers of cells were seeded in 24-well plates and counted at the times indicated (where d0 is day 0, etc.). One hundred micromolar 8-Cl-cAMP or 50 μM PD98059 (PD) was administered 6 h after plating. Medium and, where appropriate, drugs were changed after 3 days. The data represent the average of four independent experiments. (B) Morphological effects of 8-Cl-cAMP and PD98059. Confluent cultures were exposed to 100 μM 8-Cl-cAMP or 50 μM PD98059 and photographed 24 h later at a magnification of ×250. (C) 8-Cl-cAMP inhibits anchorage-independent growth of v-abl- and v-raf-transformed cells. Cells were suspended in soft agar. The agar was overlaid with medium containing 8-Cl-cAMP as indicated. The overlay was replenished every 3 days. Pictures were taken on day 10.

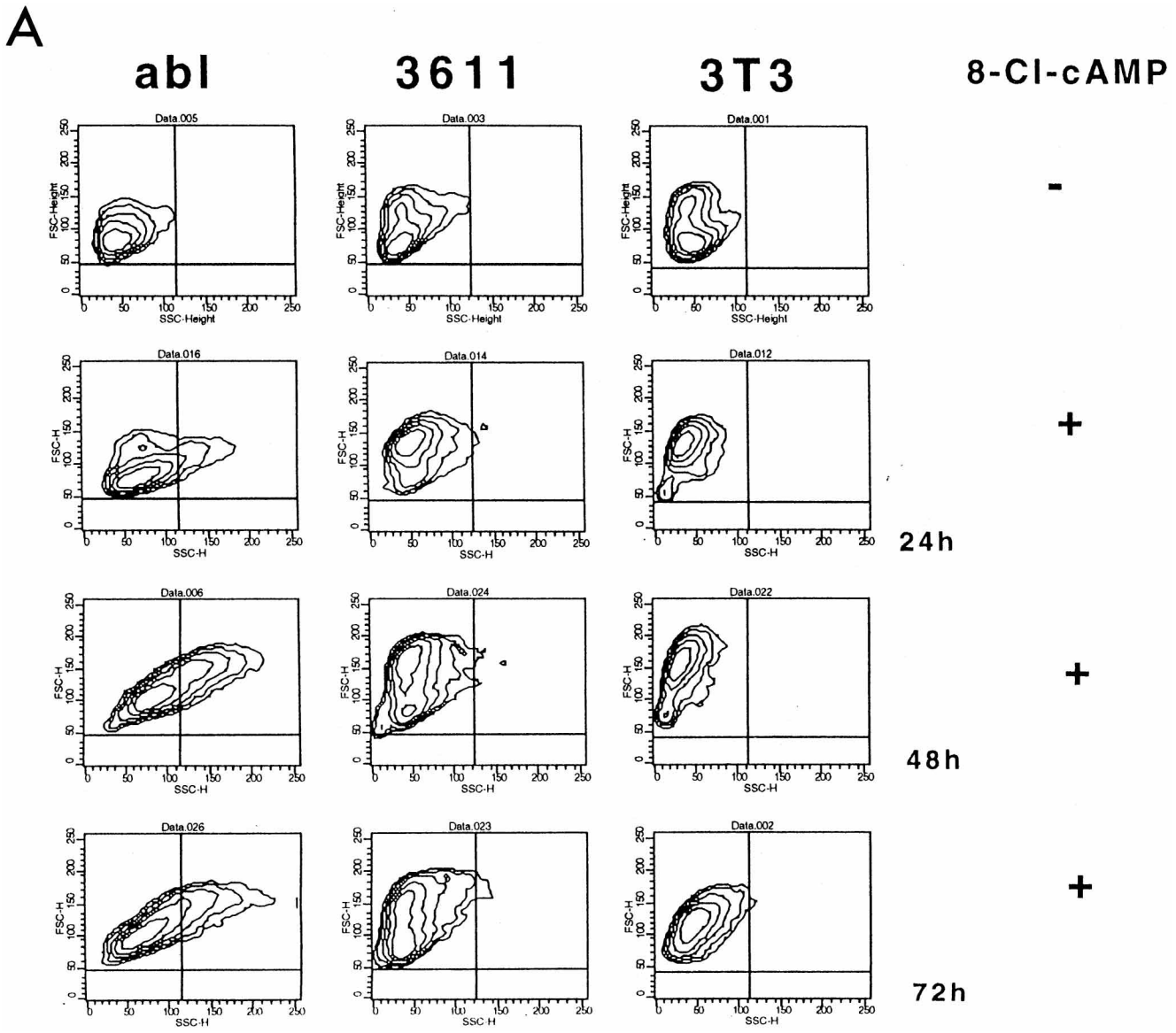
chambers, the slides were mounted and subjected to microscopic analysis. The values for percentages of apoptotic cells relative to total number of cells shown in this paper were obtained by counting at least five microscopic fields containing an average of 100 cells per field. Nuclear condensation as shown by DAPI staining in the absence of trypan blue uptake is considered characteristic of apoptotic cell death as opposed to necrotic cell death.

Immunoprecipitation, immune complex kinase assays, and Western blotting. Cells were treated as indicated in the figure legends, washed with PBS, and lysed in TBST buffer (20 mM Tris HCl [pH 7.4], 150 mM NaCl, 2 mM EDTA, 1% Triton X-100) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 1 μg of leupeptin per ml) and phosphatase inhibitors (20 mM β-glycerophosphate, 2 mM sodium fluoride, and 2 mM sodium pyrophosphate). Cell lysates were cleared by centrifugation at 20,000 × *g* for 10 min and incubated with anti-Raf, anti-MAPK, or anti-abl antiserum plus protein A-agarose (Boehringer) for 2 h. Immunoprecipitates were washed three times in TBST and once with Raf kinase buffer (20 mM Tris HCl [pH 7.4], 20 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol). Raf kinase reaction mixtures contained 100 ng of purified recombinant kinase-negative Mek or, in the case of linked kinase assays, 50 ng of kinase-competent Mek plus 150 ng of kinase-negative MAPK (33), 2 μM ATP, and 5 μCi of [γ-^{32}P]ATP. MAPK was assayed with 1 μg of myelin basic protein (Gibco/BRL) in the presence of 20 μM ATP and 2 μCi of [γ-^{32}P]ATP. After incubation for 30 min at 25°C, reactions were terminated by boiling in sodium dodecyl sulfate (SDS)-gel sample buffer and the products were separated by SDS-gel electrophoresis and the products were and autoradiographed. v-abl immunoprecipitates were resuspended in tyrosine kinase buffer (50 mM Tris HCl [pH 7.6], 10 mM $MgCl_2$, 2 mM $MnCl_2$) and incubated with 5 μg of acid-denatured enolase (Sigma), 2 μM ATP, and 5 μCi of [γ-^{32}P]ATP for 20 min. Western blotting was carried out as described previously (38) with an enhanced chemiluminescence kit (Amersham) for detection.

Northern blotting. Cells were grown to subconfluence in 10-cm-diameter tissue culture plates (Nunc) and serum starved overnight. The next day the cells were stimulated with 20% FCS for 0, 15, 90, and 300 min. In an identical set of experiments, 8-Cl-cAMP was added 30 min before stimulation. Total RNA was prepared with an RNeasy total RNA preparation kit (Qiagen) according to the manufacturer's protocol. Five micrograms of each RNA was fractionated on a 1.25% agarose gel containing 20% formaldehyde. The RNA was transferred to a Hybond-N membrane (Amersham) by capillary blotting and hybridized with a ^{32}P-labeled 1.3-kb *Xho*I fragment of pMmyc54, a probe specific for murine c-myc (88). Radioactive labeling was performed with the random primers DNA labeling system (Gibco/BRL) by following the manufacturer's instructions. The membranes were hybridized overnight, washed twice with 2× SSC at room temperature and once with 0.1× SSC at 65°C, and exposed to X-ray film (Kodak XAR-5) overnight at −80°C with intensifying screens.

RESULTS

Regulation of Raf-1 and MAPK in v-abl-transformed cells. Based on the rationale outlined in the introduction, we first examined whether the Raf-1–MAPK pathway was activated in v-abl-transformed cells. In these cells the level of the endogenous Raf-1 protein was elevated approximately 2-fold compared to that of parental NIH 3T3 cells, but its catalytic activity was enhanced more than 20-fold (Fig. 1A). Raf-1 isolated from v-abl-transformed cells readily reacted with antiphosphotyrosine antibodies, suggesting that v-abl activated Raf-1 via phosphorylation on tyrosine residues. The kinase activity of the endogenous Raf-1 protein in v-abl-transformed cells was constitutive and approached the activity observed in Raf immunoprecipitates from v-raf-transformed cells, which contain both v-raf and Raf-1 (Fig. 1B). Previously, we have shown that PKA can suppress the activity of v-raf by direct phosphorylation of the kinase domain. As a consequence, v-raf was unable to activate the Mek-MAPK signalling cascade (38).



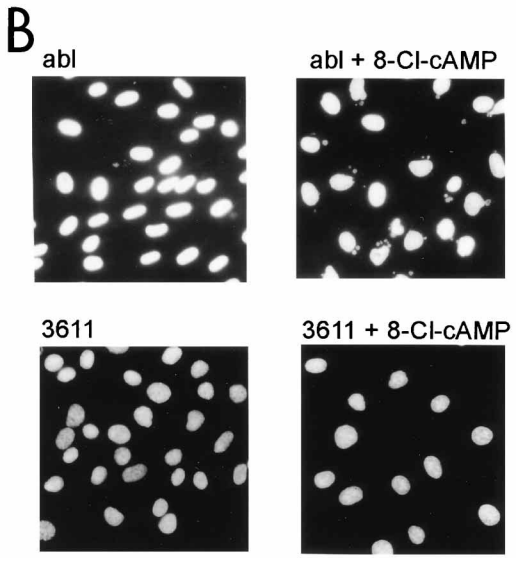


FIG. 3. 8-Cl-cAMP induces apoptosis in v-abl-transformed cells. (A) FACS analysis. Logarithmically growing v-abl-transformed, 3611, and NIH 3T3 cells were exposed to 100 μM 8-Cl-cAMP and examined for apoptosis by flow-cytometric analysis at the time points indicated. As is apparent from the increase in side scatter (*x* axis), v-abl-transformed cells undergo apoptosis in response to 8-Cl-cAMP whereas v-raf and NIH 3T3 cells do not. The control (without 8-Cl-cAMP) shows untreated cells harvested after 72 h. No signs of apoptosis were detected in untreated cells at the earlier time points. SSC-H, side scatter, height; FSC-H, forward scatter, height. (B) DAPI staining. Cells were grown on coverslips and treated with 8-Cl-cAMP where indicated. The cells were subsequently stained, and photographs were taken at a magnification of ×630. Treatment with 8-Cl-cAMP caused the characteristic apoptotic changes in nuclear morphology (chromatin condensation and nuclear disintegration) in v-abl-transformed cells but not in v-raf-transformed 3611 cells.

To examine the status of the Raf-Mek-MAPK pathway in v-abl- and v-raf-transformed (3611) cells after activation of PKA, cells were treated with the nonhydrolyzable cAMP analog, 8-Cl-cAMP, and the kinase activities of Raf-1 and MAPK were tested (Fig. 1B). In growing NIH 3T3 cells, Raf-1 immunoprecipitates exhibited little kinase activity towards Mek. The residual activity could be virtually abolished by treatment with 8-Cl-cAMP. As expected, v-raf-transformed cells displayed

TABLE 1. Quantitation of apoptosis

Treatment, time (h)[a]	% Apoptosis[b] with cell line:		
	v-abl-transformed	3611	NIH 3T3
FACS analysis (side scatter)			
None, 24	3.4	2.0	1.2
None, 72	3.9	3.2	1.9
8-Cl-cAMP, 24	24.6	3.6	4.6
8-Cl-cAMP, 48	33.5	3.8	3.5
8-Cl-cAMP, 72	50.1	4.6	5.6
BIMPS, 72	50.5	5.7	3.1
PD98059, 24	6.4	1.6	2.5
PD98059, 72	7.7	3.6	3.2
DAPI staining			
None, 24	2.0	1.8	2.1
None, 48	5.9	3.0	2.4
8-Cl-cAMP, 24	18.1	2.5	2.5
8-Cl-cAMP, 48	34.2	3.3	4.0
PD98059, 24	4.2	1.2	1.0
PD98059, 48	6.8	2.5	2.4

[a] Cells were treated with either 100 mM 8-Cl-cAMP, 100 mM Sp 5,6-DCl-c-BIMPS (BIMPS), or 50 μM PD98059 at the indicated time points.

[b] Apoptosis was calculated based on the data obtained by FACS analysis shown in Fig. 3 and after counting apoptotic cells after DAPI staining. Apoptosis is given as a percentage of apoptotic cells.

high Raf kinase activity and constitutive activation of MAPK. 8-Cl-cAMP substantially reduced the v-raf activity and subsequent activation of p44 and p42 MAPK (Erk-1 and -2). 8-Cl-cAMP also completely suppressed the activity of Raf-1 in v-abl-transformed cells. 8-Cl-cAMP did not alter the activity of the v-abl kinase, suggesting that this compound did not impair v-abl signalling directly. Interestingly, in v-abl-transformed cells 8-Cl-cAMP had no significant influence on the activity of MAPK (Fig. 1B), which appeared constitutively activated.

There is increasing evidence that p42 and p44 MAPK can be activated independently of Raf-1 (5, 43, 46, 49, 55, 66, 77). Therefore, the influence of 8-Cl-cAMP on MAPK activation in v-abl-transformed cells was monitored in more detail with time course experiments (Fig. 1C). Levels of MAPK activity were compared in serum-starved v-abl-transformed cells which had been stimulated with FCS in the presence or absence of 8-Cl-cAMP. FCS was able to superinduce the constitutive activity of Erks in v-abl-transformed cells. 8-Cl-cAMP reduced both the amplitude and duration of MAPK activation but clearly did not abolish it. These results indicate that Raf-1 contributes to MAPK activation triggered by v-abl but is not exclusively responsible for it. They also suggest that the inhibition of v-abl transformation by PKA was not due to the obstruction of the Mek-MAPK pathway.

Since the literature on the effect of PKA on B-Raf is controversial and, hence, B-Raf might be responsible for PKA-insensitive activation of the MAPK pathway in v-abl-transformed cells, we examined the cells for the presence and activity of B-Raf, using COS cells transiently transfected with a B-Raf expression vector (41) as a positive control. B-Raf was undetectable in all cells examined on Western blots of either crude cell lysates or even immunoprecipitates (Fig. 1D). In addition, the kinase activity of B-Raf immunoprecipitates was examined in a linked kinase assay with Mek and kinase-negative Erk as substrates. The kinase activity recovered from 3611 as well as the parental NIH 3T3 cells was not elevated above that of the background control prepared with preimmune serum. The small increase in kinase activity in the v-abl-transformed cells might either be due to minute amounts of B-Raf protein, undetectable on a Western blot, or due to a nonspecific reaction of the B-Raf antibody.

PKA activation inhibits v-raf and v-abl transformation. To compare the biological consequences of Raf inhibition, parental NIH 3T3 fibroblasts as well NIH 3T3 cells transformed by v-raf or v-abl were treated with 8-Cl-cAMP. To further examine the effect of the inhibition of the MAPK pathway, the cells were also treated with the specific Mek inhibitor PD98059. 8-Cl-cAMP slowed the proliferation rate of NIH 3T3 cells but completely abolished the growth of v-raf and v-abl transformants (Fig. 2A). In the experiment shown, the medium was replenished after 3 days and the drugs were readministered. This was necessary, since we observed a substantial increase in cell numbers between days 3 and 4 in 8-Cl-cAMP-treated 3611 cells, which could even regain their full proliferative capacity after a single dose of 8-Cl-cAMP (data not shown). This recovery of the proliferation rate could be completely prevented by supplying fresh 8-Cl-cAMP on day 3, suggesting that it was due to the degradation of the substance. In contrast, v-abl-transformed cells did not recover, indicating that 8-Cl-cAMP had inflicted irreversible damage selectively on these cells. In contrast to 8-Cl-cAMP, PD98059 had little or no effect on the v-abl cells but lowered the proliferation rate of NIH 3T3 and 3611 cells.

Examination of the morphology showed that 8-Cl-cAMP-treated 3611 cells flattened, and cells gained a more ordered growth pattern, indicative of reversion of transformation (Fig. 2B). In contrast, 8-Cl-cAMP induced the formation of long spiny cellular extensions in v-abl-transformed cells but few changes in the overall morphology. The cell number was consistently reduced, however. While treatment of the 3611 cells with PD98059 essentially reproduced the morphological

TABLE 2. Cell cycle analysis of 8-Cl-cAMP-treated cells

Cell line	Time of harvest (h)[a]	Treatment with 8-Cl-cAMP[b]	% of cells in phase[c]:		
			G_0/G_1	S	G_2/M
NIH 3T3	0	−	49	42	6
	24	−	44	45	11
	48	−	94	1	4
	24	+	81	10	9
	48	+	94	1	4
3611	0	−	34	59	5
	24	−	40	50	5
	48	−	47	42	7
	24	+	45	43	10
	48	+	53	33	10
v-abl-transformed cells	0	−	40	42	12
	24	−	54	37	6
	48	−	70	9	13
	24	+	30	27	38
	48	+	25	4	66

[a] All untreated cell lines had reached confluency after 48 h.

[b] Exponentially growing cells were treated with 100 μM 8-Cl-cAMP as indicated.

[c] After cells were labelled with bromodeoxyuridine, DNA content was analyzed by flow cytometry. Shown are the percentages of cells in a particular phase of the cell cycle at the time of harvest.

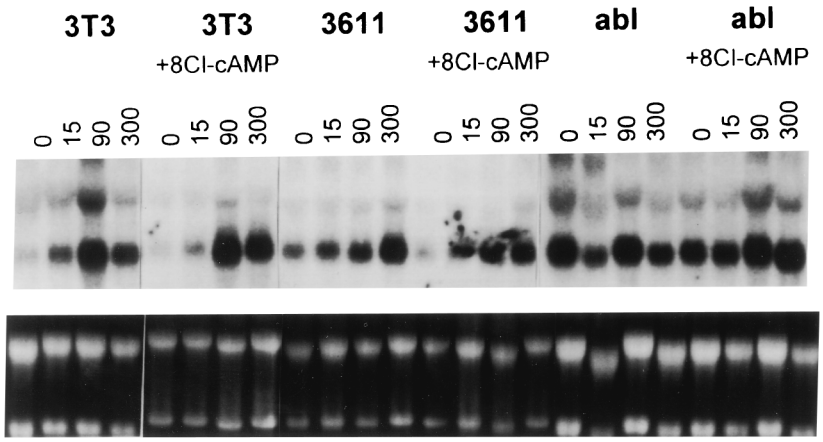


FIG. 4. v-abl-transformed cells continue to express high levels of c-*myc* RNA in the presence of 8-Cl-cAMP. Serum-deprived cells were treated with 20% FCS for 15, 90, and 300 min. Where indicated, 100 μM 8-Cl-cAMP was administered 30 min before stimulation. Total RNA was prepared and examined for c-*myc* expression by Northern blotting. To control for differences in loading, the ethidium bromide stain of the gel is included. In NIH 3T3 and v-raf-transformed cells, addition of serum induced expression of c-*myc*, while it did not augment the constitutive high expression of c-*myc* in v-abl-transformed cells. 8-Cl-cAMP did not downmodulate expression of c-*myc* in v-abl-transformed cells.

changes observed in the presence of 8-Cl-cAMP, this was not the case for the v-abl-transformed cells. Addition of PD98059 resulted, as expected, in a partial reduction of the transformed phenotype, but the formation of the long spiny cellular extensions that are typically observed after treatment with PKA activators could not be detected. Further, as already evident from the growth curves, the number of cells was not significantly reduced. To test a more stringent parameter of fibroblast transformation, cells were monitored for anchorage-independent growth in soft agar. 8-Cl-cAMP completely suppressed the colony formation of v-raf- and v-abl-transformed cells (Fig. 2C), demonstrating that PKA activation abrogates transformation by both oncogenes. Consistent results were obtained with other agents which elevated cAMP production in cells, such as forskolin, or agents which activated PKA directly, such as 8-bromo-cAMP, CTP-cAMP, Sp 5,6-DCl-c-BIMPS, and dibuturyl-cAMP. 8-Cl-cAMP and Sp 5,6-DCl-c-BIMPS proved to be the most stable compounds in cell culture medium, and therefore 8-Cl-cAMP was used routinely.

PKA activation induces apoptosis in v-abl-transformed cells. The biological and biochemical assays pointed to a fundamental difference between the actions of 8-Cl-cAMP in v-raf- and v-abl-transformed cells. A striking observation was that 8-Cl-cAMP reduced the cell number of v-abl transformants without obvious reversion of their transformed morphology. In contrast, 8-Cl-cAMP at least partially reverted the transformed morphology of 3611 cells and retarded their proliferation rate. This prompted us to investigate whether 8-Cl-cAMP induced apoptosis in v-abl transformants (Fig. 3). Apoptosis was determined by a flow-cytometric method based on the observation that apoptotic cells are characterized by nuclear condensation, membrane blebbing, and formation of apoptotic bodies. These events result in an increase of the side scatter, which correlates with the extent of apoptosis in a cell population, and thus allow us to quantitate the fraction of cells succumbing to apoptosis (13, 17, 22, 30, 90). 8-Cl-cAMP markedly enhanced apoptosis in v-abl-transformed cells, while v-raf transformants or parental NIH 3T3 cells were spared. Apoptosis of v-abl cells increased progressively (Table 1). On day 4 after exposure to 8-Cl-cAMP, almost all of the v-abl cells had died whereas parental NIH 3T3 and 3611 cells still did not exhibit apoptosis above the background rate. It should be noted that a single dose of 8-Cl-cAMP was sufficient to almost completely eliminate the population of v-abl-transformed cells. This means that the apoptotic program must have been irreversibly initiated within the first 48 h of treatment, since under cell culture conditions the efficacy of 8-Cl-cAMP is exhausted after this time.

To eliminate possible artifacts due to effects of metabolic products of 8-Cl-cAMP, we used a different PKA activator, Sp 5,6-DCl-c-BIMPS. Again, 50% of the v-abl-transformed cells scored as apoptotic after 72 h, while no significant amount of apoptotic cells could be observed in the 3611 and the parental NIH 3T3 cells. In contrast to the PKA activators, inhibition of Mek did not result in a significant amount of apoptotic cells, as could be expected from the data presented in Fig. 2.

To further substantiate our findings, we also used DAPI staining as an independent method to examine and quantitate the degree of apoptosis induced. As also shown in Table 1, the number of apoptotic cells observed by this method correlated exceedingly well with the number of apoptotic cells observed by fluorescence-activated cell sorter (FACS) analysis.

In macrophages, PKA activation was shown to cause cell cycle arrest in the G_1/S phase by inducing $p27^{kip}$, an inhibitor of G_1/S-specific cyclin-dependent kinases (47). Therefore, logarithmically growing parental NIH 3T3 cells and v-raf- and v-abl-transformed cells were analyzed for cell cycle progression in response to 8-Cl-cAMP treatment (Table 2). 8-Cl-cAMP efficiently arrested NIH 3T3 cells in G_1 phase to a degree similar to that seen in cells that became quiescent because they had grown to confluency (48-h time point). 3611 cells also slowly started to accumulate in G_1, as they approached saturation density. This accumulation was slightly accelerated by 8-Cl-cAMP. In addition, the drug increased the fraction of 3611 cells in G_2. v-abl-transformed cells were also retained in G_1 phase when they reached saturation density (after 48 h). 8-Cl-cAMP led to a pronounced accumulation of v-abl-transformed cells in G_2, suggesting that 8-Cl-cAMP triggers apoptosis by preventing v-abl-transformed cells from traversing the G_2/M phase. As all known cyclin-dependent kinase inhibitors function in the G_1/S phase transition of the cell cycle (recently reviewed in reference 86), these results strongly argue against their involvement as promoters of apoptosis in this setting. Moreover, 8-Cl-cAMP did not induce expression of

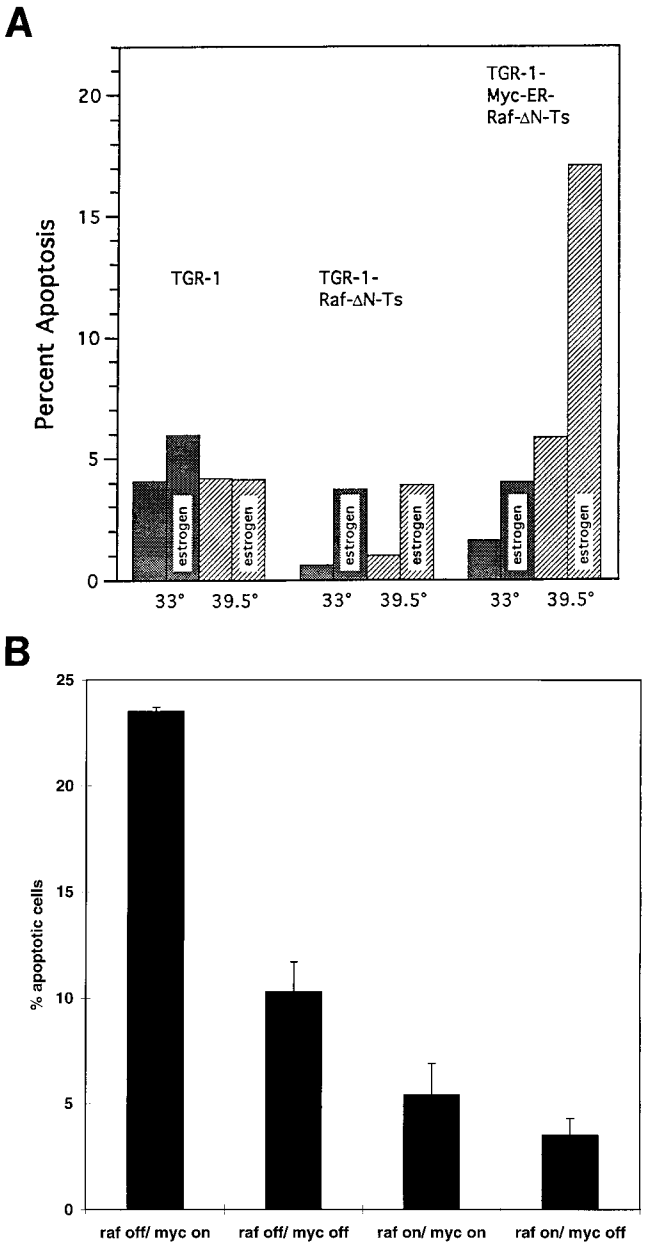


FIG. 5. Raf activation prevents c-*myc*-induced apoptosis. Apoptosis was quantitated in a cell system where c-*myc* and Raf functions can be regulated independently. Raf-ΔN-ts is a Raf-1 mutant which lacks the regulatory domain and is temperature sensitive due to point mutations in the kinase domain. It is transforming at 33°C and inactive at 39.5°C. Myc-ER is a fusion protein between c-*myc* and the hormone binding domain of the estrogen receptor. Its activity is induced by estrogen. Parental TGR-1 fibroblasts or cells which express Raf-ΔN-ts or both Raf-ΔN-ts and Myc-ER were incubated under conditions where either one or both of the transgenes were active. (A) Quantitation of apoptosis was performed by counting floating cells, which represent the apoptotic cells as evidenced by DNA laddering. In parental as well as in Raf-ΔN-ts-expressing cells, apoptosis was low under any condition. In double-expressing cells, activation of Myc-ER triggered apoptosis only when Raf-ΔN-ts was inactive. At the permissive temperature Myc-ER-induced apoptosis was completely prevented. (B) Quantitation of apoptosis in TGR-Raf^{ts}-MycER-1 cells by the terminal deoxynucleotidyl transferase-mediated dUTP nicked-end labeling assay.

p27^{kip} in NIH 3T3, 3611, or v-abl-transformed cells (data not shown).

Maintenance of constitutive c-*myc* expression in v-abl-transformed cells. A possible explanation for the different biological responses to PKA activation in v-abl-transformed cells lies in the potential of v-abl to induce high-level expression of the c-*myc* proto-oncogene (2, 8, 75, 100). c-*myc* has a dual effect. On one hand, its expression is required for Abl transformation and proliferation in response to mitogens (reviewed, e.g., in reference 82). On the other hand, c-*myc* can trigger apoptosis in all phases of the cell cycle when it is expressed in the absence of appropriate growth or survival factors (reviewed in reference 23). Therefore, the effects of 8-Cl-cAMP on the expression of c-*myc* were examined (Fig. 4). The expression of c-*myc* mRNA was regulated by serum growth factors in parental NIH 3T3 cells but was constitutively elevated in 3611 (v-raf-transformed) and v-abl-transformed cells. In 3611 cells, c-*myc* mRNA levels could be further increased by serum stimulation, whereas c-*myc* could not be superinduced in v-abl-transformed cells, indicating that c-*myc*'s expression was already maximally activated. 8-Cl-cAMP did not affect the high expression of *myc* in v-abl-transformed cells, whereas it reduced constitutive expression in 3611 cells. This reduction probably was a secondary consequence of the reduction of cell proliferation following v-raf inhibition, as there is no evidence that v-raf can regulate c-*myc* expression. However, in v-abl-transformed cells, 8-Cl-cAMP led to the inhibition of Raf-1 function, while high-level expression of c-*myc* persisted. This constellation might correspond to the situation where c-*myc* expression in the absence of growth factor signalling triggers apoptosis. Therefore, we tested whether Raf-1 activation could protect cells from myc-induced apoptosis.

Activation of a temperature sensitive Raf-1 mutant prevents *myc*-induced apoptosis. Raf-1 activation as well as c-*myc* expression is caused by a wide variety of extracellular stimuli which have pleiotropic effects on cells. To study the specific contribution of individual components, it is necessary to utilize conditional mutants or expression systems. Therefore, we employed rodent TGR-1 fibroblasts, which had been engineered to express a temperature sensitive Raf-1 mutant, Raf-ΔN-ts, and an estrogen-regulated Myc-estrogen receptor fusion protein, Myc-ER. TGR-1 is a diploid subclone of the Rat-1 cell line (71), which has been used as model system for c-*myc*-induced apoptosis (15, 24, 31). The construction of Raf-ΔN-ts and the generation of cell lines are described in Materials and Methods. Myc-ER fusion proteins can be activated by estrogen (21) and have been widely used to investigate the function of the c-*myc* protein. The generation and biological properties of the Raf-ΔN-ts mutant will be described in more detail elsewhere. In brief, this mutant was derived from an N-terminally truncated version of Raf-1 (EC12) which is a transforming oncoprotein (39). Replacement of R391 and E393 with alanine residues rendered this protein temperature sensitive. It behaves like a transforming *raf* allele at the permissive temperature of 33°C but exhibits no activity at 39.5°C. As shown in Fig. 5, temperature shifts had no significant influence on the low rate of apoptosis of parental or Raf-ΔN-ts-transfected cells, thus ruling out a temperature effect. On the other hand, activation of *myc* by the addition of estrogen caused apoptosis in cells expressing Myc-ER, but no effect of estrogen could be observed in the parental cells. At the nonpermissive temperature and hence in the absence of active Raf, estrogen rapidly induced apoptosis in cells expressing both Raf-ΔN-ts and Myc-ER. Activation of Raf-ΔN-ts almost completely prevented apoptosis by Myc-ER. The protective effect of Raf-ΔN-ts was observed with two independent methods of assaying apoptosis. These data suggest that Raf-1 can provide a survival signal which antagonizes c-*myc*-induced apoptosis.

Overexpression of Raf-1 in v-abl-transformed cells leads to partial insensitivity towards PKA activation. As we have de-

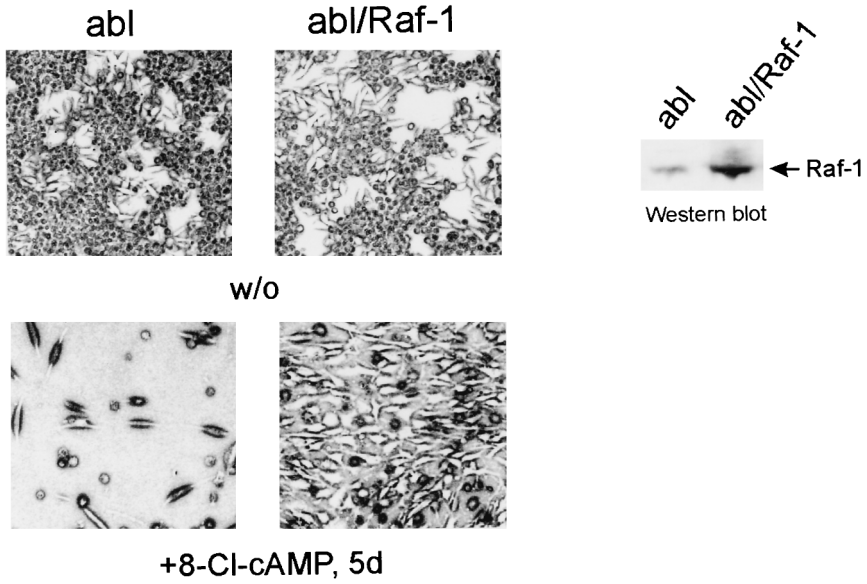


FIG. 6. Overexpression of Raf-1 in v-abl-transformed cells leads to partial insensitivity towards PKA activation. v-abl-transformed cells were transfected with a Raf-1 expression vector and selected with G418. G418-resistant colonies (abl–Raf-1) were pooled and analyzed for their response to 8-Cl-cAMP. Subconfluent cultures of abl–Raf-1 and parental v-abl-transformed cells were either left untreated (w/o) or exposed to 100 μM 8-Cl-cAMP (+8-Cl-cAMP, 5d) and photographed 5 days later at a magnification of ×100. The photograph on the right shows a Western blot stained with the crafVI antiserum. Equal loadings were confirmed by staining of the blot with PonceauS prior to incubation with the antibody.

scribed previously (65), phosphorylation of serine 621 in Raf-1 by PKA renders Raf-1 kinase kinase inactive. However, mutation of this site also results in a kinase-negative Raf-1. Hence, we were unable to overexpress a PKA-insensitive, kinase-active Raf-1 mutant to give absolute proof that Raf-1 inactivation leads to the above-described effects. Assuming that overexpression of wild-type Raf-1 might result in a partial insensitivity towards PKA, we transfected the v-abl-transformed cells with the Raf-1 expression vector. As shown in Fig. 6, we were able to express Raf-1 ca. fivefold over the endogenous level. In the absence of 8-Cl-cAMP, the v-abl–Raf-1 cells were essentially indistinguishable from the parental v-abl-transformed cells. In the presence of the PKA activator, however, we could observe obvious differences. While the v-abl-transformed cells declined in numbers due to increased apoptosis and only a few cells were present after 5 days, the v-abl–Raf-1 cells revealed changes in their morphology and growth retardation, but apoptosis was clearly reduced (Fig. 6). As anticipated, overexpression of wild-type Raf-1 could not completely protect the cells from PKA-induced growth inhibition. However, these experiments strongly support the notion that inhibition of Raf-1 by PKA is responsible for the induction of apoptosis in the v-abl-transformed cells.

DISCUSSION

In this report we have investigated the biological consequences of the suppression of Raf-1 signalling by PKA activation in v-abl-transformed cells. The observation that Raf-1 was phosphorylated on tyrosine residues in v-abl-transformed cells suggests that v-abl activates Raf-1 by direct phosphorylation. Raf-1 activation due to phosphorylation by src family tyrosine kinases has been previously observed in vitro as well as in Sf-9 insect cells and T cells (26, 38, 94, 99). Recent observations indicate a physiological role for src family kinases in the activation of Raf-1 in fibroblasts (26, 42, 60). Our results demonstrate that a different class of tyrosine kinases represented by v-abl can function as efficient Raf-1 activators.

Raf-1 activation also seems to be required for transformation by v-abl, as PKA-mediated inhibition of Raf-1 results in apoptosis. Furthermore, overexpression of Raf-1 in v-abl-transformed cells severely impairs the induction of apoptosis

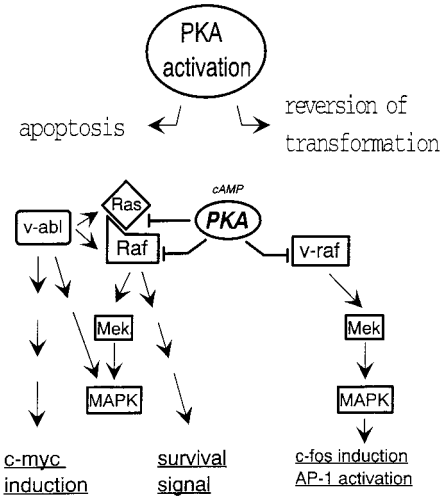


FIG. 7. Model for the differential effects of Raf inhibition in v-raf- and v-abl-transformed cells. v-raf transforms cells by constitutively activating the Mek-Erk pathway. PKA inhibition of v-raf inhibits the transmission of the transforming signal, resulting in reversion of transformation and a reduced proliferation rate. In contrast, v-abl transformation involves Raf-1 activation as well as constitutive expression of c-*myc*. v-abl may contribute to Raf-1 activation both by inducing GRNP to activate Ras and by phosphorylation of Raf-1. Inhibition of Raf-1 by PKA precipitates apoptosis, probably because it deprives the cells of a survival signal which complements the sustained expression of c-*myc*. This Raf-1 signal cannot be substituted by Erks, whose activities remain elevated despite Raf-1 inhibition.

by PKA. It cannot be completely excluded that PKA contributes to apoptosis by a Raf-1-independent pathway as well, although this possibility appears unlikely. PKA can stall the cell cycle in G_1 by inducing $p27^{kip}$, an inhibitor of cyclin-dependent kinases (47). However, this mechanism does not seem to be responsible for the accumulation of 8-Cl-cAMP-treated NIH 3T3 cells in G_1. $p27^{kip}$ is expressed at low levels in all the cell lines studied here, but its expression is not affected by cAMP agonists (data not shown). Furthermore, 8-Cl-cAMP-treated v-abl-transformed cells die in G_2 phase, excluding the involvement of other known cell cycle inhibitors, which act in G_1/S (reviewed, e.g., in reference 86). On the other hand, Src, an upstream activator of Raf-1, is known to be activated in G_2/M (3, 32, 93). Src (78) as well as Ras (20) seem to be required for entry into mitosis. Recent data report Raf-1 activation in G_2/M (59), suggesting that Raf inhibition by PKA may delay mitosis and contribute to the reversion of v-raf transformation.

In v-raf-transformed cells, however, Raf inhibition results in growth retardation and reversion of transformation. The potential basis for this difference might rest in the ability of v-abl to induce the c-*myc* gene. The activation of expression of c-*myc* seems to be essential for transformation by v-abl, since dominant negative *myc* mutants can block v-abl transformation of pre-B cells and fibroblasts (83). c-*myc* expression is a prerequisite for cells to progress into S phase and is also induced during transition from S to G_2 (85). On the other hand, c-*myc* expression in growth factor-deprived cells can cause apoptosis in any stage of the cell cycle, indicating that v-abl must in addition provide for the rescue of cells from *myc*-induced apoptosis. v-abl efficiently abrogates growth factor requirements (8, 51) and can protect cells from apoptosis elicited by fas (90), growth factor withdrawal (25, 37, 45), or drug treatment (7). Therefore, it appears plausible that v-abl couples to intracellular signalling molecules, thus supplying a survival function.

Besides c-*myc* induction, the activation of Ras has been shown to be required for transformation by oncogenic Abl proteins (35, 70, 84). Furthermore, the antiapoptotic effect of Bcr-Abl (and most likely v-abl) requires the association of Bcr-Abl with Ras (12) and hence most likely the activation of Raf-1. v-abl and Bcr-Abl can associate with shc, grb-2, and crk adaptor proteins, which mediate ras activation via the guanine nucleotide-exchange factors SOS and C3G (25, 28, 74, 76). A main target of Ras is the Raf-1 kinase, which can activate the Mek-Erk cascade (reviewed in references 18, 61, 68, and 98). The fact that Raf-1 inhibition in v-abl-transformed cells only partially dampened the activity of Erk-1 and -2 indicates that v-abl entertains an alternative Raf-1-independent pathway to signal these kinases. This pathway may be cell-type specific, since in COS cells v-abl could activate Erk-1 in transient transfection experiments and Erk-1 activation could be blocked by cotransfection of a dominant negative Raf-1 mutant (95). Regardless of the role of Raf-1 in MAPK activation by v-abl, our data suggest that the Raf-1 kinase might supply an important survival signal in v-abl-transformed cells. Several lines of evidence support this conclusion. There is no indication that Raf-1 or v-raf mediate c-*myc* induction. v-raf and v-myc, however, exhibit a strong synergism in terms of transformation and growth factor abrogation, pointing to a mutual functional complementation (9, 51, 72). Moreover, v-raf can reduce apoptosis in interleukin 3 (IL-3)-dependent 32D cells following IL-3 withdrawal (10). Using cells which express conditional versions of Myc and transforming Raf, we could show in this study that Raf activation can prevent Myc-induced apoptosis. This protective function of Raf-1 must be mediated by a MAPK-independent pathway, since MAPK activity in v-abl-transformed cells did not correlate with the extent of Raf-1 inhibition. In accordance with this notion, Owen Lynch et al. found in an IL-3-dependent cell line that suppression of apoptosis by v-abl upon growth factor removal does not require MAPK (69). Recently, evidence was reported that Raf-1 may protect cells from apoptosis by phosphorylating and inactivating the apoptosis-promoting bcl-2 family member BAD (97, 102). This mechanism is completely independent of the activation of the MAPK pathway and hence might well explain our findings.

While this paper was under revision, Kauffmann-Zeh et al. (48) reported that a membrane-targeted version of Raf-1 (Raf-CAAX) activates Erks and promotes apoptosis. Our results too suggest that Erk activation is not critical for prevention of apoptosis but that Raf-1 activity reveals a protective effect independent of Erks. This is in accordance with the results of Wang et al. (97), who showed that Raf-CAAX activates Erks but fails to prevent apoptosis while wild-type Raf-1 protects cells. These and our results indicate that Raf-1 functions in more than one pathway and suggest that the antiapoptotic function of Raf-1 may be precluded after constitutive membrane targeting.

In conclusion, we propose that Raf-1 participates in v-abl transformation by rescuing cells from the potentially deleterious effects of c-*myc* activation. A model summarizing our findings is presented in Fig. 7. In addition, the ability of cAMP agonists to induce apoptosis of certain tumor cells may lend itself to the development of novel strategies for tumor therapy. In this respect, the ability of 8-Cl-cAMP to inhibit the growth of experimental tumors in nude mice (1) provides an intriguing lead.

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