Subcellular Localization Determines the Protective Effects of Activated ERK2 against Distinct Apoptogenic Stimuli in Myeloid Leukemia Cells*

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ERKs, mitogen-activated protein kinases, are well characterized as key mediators in the conveyance of signals that promote cell survival in cells of hemopoietic origin, a key factor in the upbringing of leukemogenesis. It is also well known that ERKs phosphorylate a wide array of substrates distributed throughout distinct cellular locations such as the nucleus, cytoplasm, and cell periphery, but the relative contribution of these compartmentalized signal components to the overall survival signal generated by activation of ERKs has yet to be established. To this end, we have utilized constitutionally activated forms of ERK2, whose expression is restricted to the nucleus or to the cytoplasm, to investigate the consequences of compartmentalized activation of ERK in the survival of chronic myelogenous leukemia cells subjected to distinct apoptogenic stimuli. We show that cytoplasmic ERK2 activation protected against apoptosis caused by prolonged serum starvation, whereas ERK2 activation restricted to the nucleus antagonized apoptosis induced by the Bcr-Abl inhibitor STI571. On the other hand, neither cytoplasmic nor nuclear ERK2 activities were effective in counteracting apoptosis induced by UV light. These results demonstrate that the protective effects of ERK2 against defined apoptotic stimuli are strictly dependent on the cellular localization where ERK activation takes place. Furthermore, we present evidence suggesting that the complex IκB-NFκB participates on ERK2-mediated survival mechanisms, in a fashion dependent on the cellular location where ERK2 is active and on the causative apoptogenic stimulus.

Most hemopoietic disorders exhibit a broad spectrum of molecular malfunctions that alter the mechanisms, one way or another, by which cells control critical processes such as proliferation, differentiation, and cell survival. Many of these alterations arise in components of the molecular circuitry through which external cues in the form of cytokines, growth factors, or hormones regulate the aforementioned biological processes. A large number of these faulty signaling intermediaries impinge, either directly or indirectly, on signal transduction pathways mediated by mitogen-activated protein kinases (MAPKs) and have profound effects on the biological outputs mediated by these signaling modules (1).

MAPKs are cytoplasmic serine/threonine kinases that are activated in response to a wide array of extracellular stimuli, including those that regulate cell proliferation, differentiation, cell survival, development, and inflammation. MAPKs are pivotal elements in the transduction of signals from the cell surface, acting as essential mediators in signaling cascades that include sequentially: MAPK kinase kinases, dual-specificity MAPK kinases, and MAPKs (1). Extracellular signal-regulated kinases (ERKs) p44 ERK1 and p42 ERK2 MAPKs are activated in response to most, if not all, stimuli that promote proliferation and/or survival. Such activation is mainly achieved through a route that includes Raf-1 serine/threonine kinase and MEK1/2 dual specificity kinase, being regulated upstream by the small GTPase Ras (1, 2). It has been proposed that the duration and amplitude of ERK signals are determinant in the decision of cell fate (3–5). As such, unregulated activation of the ERK pathway is common in human neoplasia, both in solid tumors (6–9) and in hemopoietic malignancies (10–12). Thus, much attention is currently being focused on the ERK pathway as a potential target for antineoplastic therapy (13).

The role of ERKs in hemopoiesis and leukemogenesis is a hot field of research. It is now widely documented that ERKs play an essential role in proliferation and differentiation in many cell types (5, 14–20). Focusing in the hemopoietic system, it is also well known that ERKs are pivotal elements in the processes regulated by multiple cytokines involved in normal hemopoiesis (21). However, their relevance in the regulation of proliferation and differentiation in malignant cells of myeloid origin is still not fully defined. In this respect, some studies, generally undertaken on established cell lines, suggest that ERK activation is critical for proliferation and differentiation (22–25). Others, however, report that leukemic cells can proliferate and differentiate irrespective of the degree of activation of the ERK pathway (10, 26, 27).

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1 The abbreviations used are: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; MBP, myelin basic protein; DTT, dithiothreitol; CML, chronic myelogenous leukemia; PARP, poly (ADP-ribose) polymerase; EMSA, electrophoretic mobility shift assay; PBS, phosphate-buffered saline; CREB, cAMP-response element-binding protein.
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Another key facet in the upbringing of malignancy is the unregulated maintenance of signals that promote survival and/or prevent apoptosis, whereby efficient removal of damaged or transformed cells can be avoided. In this respect, several lines of evidence indicate that the balance among the intensity and duration of pro- versus anti-apoptotic signals transmitted by the different MAPK modules is a determinant in the decision whether a cell survives or undergoes apoptosis (1). In most cell types tested, the activation of ERKs provides a strong survival signal. Thus, blocking the ERK pathway has proved to be an efficient mechanism to force cells into apoptosis (28, 29). This strategy, generally practiced by the use of MEK1/2 pharmacological inhibitors PD98059 (30), U0126 (31), and PD184352 (32), has been useful as a means to potentiate the anti-tumoral effects of various cytotoxic agents, including cisplatin (33), ara-C (34), and taxol (35). In this respect, the inhibition of ERK activity has been shown recently to be particularly effective in enhancing apoptosis in chronic myelogenous leukemia (CML) cell lines by acting in synergy with the drug STI571 (36), a pharmacological inhibitor for the chimeric protein kinase Bcr-Abl, present in over 95% of CML cases (37, 38). However, the molecular mechanisms by which ERK activity levels have an influence on Bcr-Abl anti-apoptotic functions are largely unveiled.

Even though a large body of data supports the involvement of the ERK pathway in the conveyance of survival signals, little is known about the downstream components and molecular mechanisms whereby activated ERKs exert their anti-apoptotic effects. To date, over 50 ERK substrates have been characterized, including protein kinases, transcription factors, exchange factors, transmembrane receptors, lipases, and structural proteins, among others. These are distributed in different cellular compartments like the nucleus, cytoplasm, plasma membrane, cytoskeleton, and mitochondria (1). The biological consequences of ERK activity confined to these distinct cellular locations and their relative contribution to ERK survival signals are largely unveiled. To this end, with the aid of constructs encoding for constitutively activated ERK2 specifically tethered to the nucleus or to the cytoplasm, we have investigated the consequences of compartmentalizing ERK activation on its ability to counteract distinct pro-apoptotic stimuli in CML cells. Here we demonstrate that the protective effects of ERK2 against distinct apoptotic stimuli are dependent on the cellular localization where its activation ensues. In this respect, we present evidence suggesting that resistance to STI571-induced apoptosis correlates proportionally with physiological nuclear ERK activity levels. Finally, we present data indicating that the interplay between the activations of ERK2 and of the complex IcB-NFkB, as a mechanism to counteract cell death, exhibits marked differences depending on the causative apoptotic stimulus.

MATERIALS AND METHODS

Cell Culture—Cells were regularly grown in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal calf serum. UO0126 was from New England Biolabs. BAY11-7082 was from Calbiochem. STI571 was a kind gift from Novartis. Routine cell growth and cell viability were assayed by hemocytometer and trypan blue exclusion test counts. Alternatively, viability was measured as metabolic activity of cells, analyzed by its capability of reducing tetrazolium salts (WST) to formazan dye. WST is reduced by NADH or NADPH coupled with an electron mediator and generates yellow-colored formazans that are measured at a wavelength of 405 nm.

Cell Transfection—Plasmids encoding for Myc-tagged ERK2/MEK1, ERK2/MEK1 LA, and their respective ERK kinase-inactive forms (39) were transfected in addition to vector plasmid harboring G418 resistance (10:1 ratio) into K562 cells by electroporation (250 V, 500 microfarads) using a Bio-Rad Gene-Pulser. After electroporation, cells were incubated for 48 h, and 0.6 mg/ml G418 was added. Individual clones were isolated by minimal dilution, and cells were selected for the following 2–3 weeks.

Immunoblotting—Leukemic cells were collected and lysed in 20 mM HEPES, pH 7.5, 10 mM EDTA, 40 mM β-glycerophosphate, 1% Nonidet P-40, 2.5 mM MgCl2, 1 mM DTT, 2 mM vanadate, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, and 20 μg/ml leupeptin. Proteins were fractionated by SDS-PAGE and transferred onto nitrocellulose filters. Immunocomplexes were visualized by enhanced chemiluminescence detection (Amersham Biosciences), using horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibodies (Cappel). Rabbit polyclonal antibodies anti-ERK1/2 and anti-phospho-ERK1/2, anti-BAD and anti-phospho-BAD, mouse monoclonal antibody anti-PARP, and rabbit polyclonal anti-RhoGDI were purchased from Santa Cruz Biotechnology. Mouse monoclonal antibodies anti-CREB and anti-phospho-CREB were from New England Biolabs. Anti-Myc mouse monoclonal was from Babco.

Kinase Assays—ERK kinase activities were determined in anti-ERK immunoprecipitates as described previously (40) by using myelin basic protein (MBP) (Sigma) or GST-Elk1 as substrates.

Electromobility Shift Assays (EMSA)—The protein-DNA complexes were analyzed by EMSA as described previously (41). Nuclear extracts (2 μg of protein) obtained from serum-starved or STI-treated cells were incubated with a 32P-labeled probe containing the NFkB-binding site.

DNA Fragmentation—DNA fragmentation was basically performed as described previously (42). Briefly, to determine the presence of internucleosomal DNA fragmentation, cells were lysed in 10 mM Tris, 1 mM EDTA, and 0.2% Triton X-100. The cytoplasmic fraction of the lysates was obtained by centrifugation, further adjusted to 150 mM NaCl, 40 mM EDTA, 1% SDS, and treated with 200 μg/ml proteinase K. DNA fragments were obtained by extraction with phenol and chloroform/isoamyl alcohol (24:1). Precipitated with ethanol and finally fractionated in 15% agarose gels containing 0.1 μg/ml ethidium bromide.

Annexin V Staining—Binding of annexin V to the cell surface was analyzed by flow cytometry, utilizing annexin V-fluorescein isothiocyanate (Genzyme Diagnostics), following the manufacturer’s instructions.

Nuclear Cytoplasmic Fractionation—Fractionation was performed essentially as described (43). Briefly, cells were collected in 50 mM β-glycerophosphate, pH 7.3, 0.2 mM EDTA, 420 mM NaCl, 1.5 mM MgCl2, 1 mM DTT, 25% glycerol, sonicated briefly on ice, vortexed, and centrifuged; the precipitated cell debris was discarded.

RESULTS

ERKs Activation Levels Inversely Correlate with Susceptibility to Apoptosis—the K562 cell line (44) is a widespread model for CML. This cell line is particularly resistant to apoptosis (45, 46), a feature attributed to the presence of Bcr-Abl (47, 48). However, K562 cells can be induced to apoptosis by treatment with cytotoxic agents such as STI571 (36) and herbimycin (49). In both cases, apoptosis correlated with a substantial drop on ERK activation levels. Prior to investigating how compartmentalizing ERK activity affects apoptosis in CML, we wanted to ascertain whether the direct relationship between ERK activation and resistance to apoptosis, as described for K562, was a general characteristic in CML cell lines. For this purpose, we utilized K562, KBM5, and KU812, typical Philadelphia-positive CML cell lines, and for comparative reasons we also included the acute myeloid leukemia cell line U937. All these

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myeloid cell lines exhibited low ERK activity levels under exponential growth conditions when compared with cell lines of epithelial or fibroblastic origin (27) (data not shown). However, among themselves there were marked differences. ERK activity levels were at least 3.8-fold higher in CML lines when compared with U937. Among the CML cell lines, K562 had the highest ERK activity levels, over 40% above those detected in KBM5 and KU812, which displayed lower ERK activation (Fig. 1A). We also investigated the resistance to apoptosis of these cell lines. To this end, cells were deprived of serum for a prolonged period, and DNA fragmentation was analyzed at different time intervals. It was found that the cell line that displayed the greatest ERK activity levels, K562, was the most resistant to apoptosis caused by deprivation of growth factors, as DNA fragmentation was only apparent after 5 days of serum starvation (Fig. 1B). On the other hand, in KBM5 and KU812 that showed lower ERK activity levels, DNA fragmentation was evident just after 2 days of starvation. In the case of KU812 cells, DNA degradation was almost complete after 5 days. The U937 cell line, in which ERK activity levels were the lowest, was the most sensitive to serum withdrawal, just after 1 day apoptosis was evident; and by the 2nd day DNA degradation was complete.

It has been show that blockade of the ERK pathway by MEK1/2 inhibitors is a potent inducer of apoptosis in K562 cells (49, 50). Thus, we tested if this was a general characteristic in CML cells irrespective of the cell line utilized. As such, KU812 and KBM5 cells were serum-starved in the presence or absence of the MEK inhibitor UO126, and apoptosis was monitored at different intervals. As shown in Fig. 1C, in KU812 cells inhibition of ERK activation resulted in an increase in DNA fragmentation, evident just after 1 day of growth factor depletion and more prominently at the 2nd day of starvation, indicating that curtailing the activity of ERK potentiated the apoptotic process. Identical results were obtained in the case of KBM5 (data not shown). Overall, these results confirmed that ERK activity levels and resistance to apoptosis correlated in all CML cell lines tested.

Compartmentalized ERK Activity Affects Cell Survival but Not Cell Proliferation—Our next step was to examine the biological effects of compartmentalizing ERK activity to restricted cellular sites. For this purpose, we utilized vectors encoding for ERK2 fused in tandem with MEK1; this fusion renders ERK2 constitutively active (39). Two versions were utilized as follows: ERK2-MEK1 (E/M), which locates to the cytoplasm, and ERK2-MEK1 LA (E/M LA), a mutant form in which four leucines in MEK1 nuclear export signal had been mutated to alanines, thereby exhibiting a predominant localization to the nucleus (39). These LA mutations did not affect the ability of this chimeric protein to interact and phosphorylate in vitro ERK

Fig. 1. ERK activation and susceptibility to apoptosis in myeloid leukemia cell lines. A, ERK activity levels in myeloid leukemia cell lines. ERK1/2 activation in exponentially growing samples of the indicated leukemia cell lines (2.5 × 10⁶ cells/sample) was determined in anti-ERK immunoprecipitates by an immunocomplex assay using MBP as substrate, as described under “Materials and Methods.” Data show an average ± S.E. of five independent experiments. B, apoptosis in leukemia cell lines induced by serum starvation. The indicated cell lines (2.5 × 10⁶ cells/sample) were cultured in the absence of serum for the indicated periods, and internucleosomal DNA fragmentation was determined as described under “Materials and Methods.” C, effects of MEK inhibitors on apoptosis induced by serum starvation. KU812 cells (2.5 × 10⁶ cells/sample) were serum-starved for the indicated periods in the absence (−) or presence (+) of UO126 (2 μM), and internucleosomal DNA fragmentation was determined as described under “Materials and Methods.”
substrates, such as MBP and Elk-1, as efficiently as E/M (Fig. 2A). K562 cells were transfected with these constructs, and individual clones were isolated on the basis of their resistance to G418. To avoid misinterpretations because of clonal peculiarities, 10 clones were selected for each construct and pooled to G418. To avoid misinterpretations because of clonal pecu-

Fig. 2. Expression of location-specific ERK/MEK fusions in K562 cells. A, interaction of ERK/MEK fusions with substrates. Myc-tagged ERK/MEK and ERK/MEK LA fusion proteins were immunopreci-

PPT. Western blot. ERK/MEK LA was capable of increasing the phosphorylation of growth factors. In K562 cells, sustained serum starva-

Overall, these data clearly suggested that compartmentalized activation of ERK2 could counteract starvation-induced apoptosis. As such, the cell lines expressing the activated ERK2 fusions were subjected to serum starvation for 5 days, and apoptosis was monitored by internucleosomal DNA fragmentation. As shown in Fig. 4A, left bottom panel, in E/M and MEK E transfectants apoptosis was markedly reduced. In comparison to these lines, in cells expressing E/M LA the extent of apoptosis was much more pronounced, although some degree of protection was also evident when compared with parental K562 cells. To ascertain this observation, we also analyzed the degree of apoptosis by other means. As such, annexin V staining yielded similar re-

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as full ERK activation, restricted to the nucleus or to the cytoplasm, affected proliferation in CML cells. The next goal was to investigate if compartmentalized ERK2 activation had an effect on CML cell survival. In order to do so, we monitored the progression of the aforementioned transfectant cell lines under serum starvation conditions. It was found that all the cell lines progressed to a similar extent up to the 2nd day of starvation, but from that point K562 parental cells and E/M LA transfectants suffered a dramatic drop in the number of viable cells. After 4 days, the number of living cells was almost three times higher in those lines expressing MEK E and E/M, despite undergoing proliferation arrest and a noticeable drop in cell viability (Fig. 3B). It was important to verify whether the survival effect observed in the E/M cell line was dependent on the ERK2 kinase activity exerted by the ERK2-MEK1 fusion. To do so, we generated a K562 cell line expressing an ERK2 dead kinase fusion (E/M LA) in which ERK2 harbored the inactivating mutation K52R in the ATP-binding site (39). By utilizing this cell line it was found that about 80% of the E/M transfectants were alive after 3 days of starvation. In contrast, despite being expressed to similar levels as E/M (data not shown), the number of living cells in the E/M LA culture dropped to 40%, a similar propor-

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activation to defined cellular locations could alter this behav-

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These results supported the notion that protection against apoptosis induced by growth factor depletion was mainly exerted by the activation of ERK2 in the cytoplasm.

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Overall, these data clearly suggested that compartmentalized activation of ERK2 did not affect the proliferation of CML cells, but it could exert a remarkably positive effect on cell survival depending on the localization where ERK activation occurred. ERK2 Activation Counteracts Distinct Apoptogenic Stimuli Depending on Its Localization—In light of the previous data, we then determined whether the observed enhanced cell sur-

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substrates, such as MBP and Elk-1, as efficiently as E/M (Fig. 2A). K562 cells were transfected with these constructs, and individual clones were isolated on the basis of their resistance to G418. To avoid misinterpretations because of clonal peculiarities, 10 clones were selected for each construct and pooled to form a mass culture in which the stable expression of the fusions was ascertained with the aid of the Myc epitope that

expression E/M LA could not (data not show). Thus, in agreement with previ-
ous results (39), our data confirmed the compartmentalized cellular distribution of these activated forms of ERK2.

Previous results from our laboratory indicate that CML cells can proliferate in a fashion independent of ERK activation (27). As such, we wished to determine whether confinement of ERK
A protective response took place in other cellular compartments. To this end, we studied the effects of compartmentalized ERK2 activation against the antileukemic drug STI571, known to be a potent inducer of apoptosis in CML cells (51). In agreement with previous results (36, 51), treatment of K562 cells with STI571 resulted in a dramatic reduction of activated ERK2 (Fig. 4B, left panel) and a pronounced apoptosis, monitored both by internucleosomal DNA fragmentation and by degradation of poly(ADP-ribose) polymerase (PARP) (Fig. 4B, right panels). Most interesting, susceptibility to STI571-induced apoptosis was only slightly affected in E/M transfectants. On the other hand, in E/M LA cells in which ERK2 activation is restricted to the nucleus, the degree of apoptosis was dramatically reduced. An antiapoptotic effect against STI571 treatment was also noticeable in the cell line expressing MEK E. In addition, we also investigated the effects of compartmentalized ERK2 activation against UV-induced apoptosis. Irradiation of leukemic cells with UV light did not result in significant changes on the activity levels of ERKs (Fig. 5A). Most striking, susceptibility to apoptosis induced by UV light was unaltered in cells where constitutive activation of ERK2 was restricted to the cytoplasm (E/M) or to the nucleus (E/M LA) (Fig. 5B), although a slight protective effect could be observed in MEK E expressors in which widespread ERK activation occurred. These results suggested that, depending on the pro-apoptotic stimulus in question, the protective response exerted by ERKs takes place in distinct cellular locations.

**Resistance to STI571-induced Apoptosis Correlates with Physiological Nuclear Phospho-ERK Levels**—The above-mentioned observations prompted us to determine whether the
resistance of a given cell line to defined apoptogenic stimuli could be dictated by the degree of ERK activation at a particular subcellular compartment. To test this hypothesis, we treated K562, KU812, and KBM5 cell lines with STI571 for up to 3 days, and their susceptibility to apoptosis was evaluated both by DNA fragmentation and by PARP degradation. As shown in Fig. 6A, it was found that KBM5 was the most sensible to STI571 treatment. Massive apoptosis was evident just after 1 day, and cell death was almost totally completed by the 2nd day of treatment. On the other hand, K562 was the most resistant to STI571-induced apoptosis, with few apoptosis signs evident during the 1st day. KU812 sensitivity to STI571 was slightly higher than that exhibited by K562; DNA fragmentation and PARP degradation were evident after the 1st day of treatment, and virtually all PARP had been degraded between the 2nd and 3rd days. Next, we evaluated whether the sensitivity to STI571 exhibited by the different cell lines could have some relationship with their respective physiological levels of activated ERKs present at their nuclear compartments. To do so, we performed nuclear-cytoplasmic fractionations for the three CML cell lines. These yielded nuclear and cytoplasmic fractions with minimal contamination, as shown by Western blotting against c-Myc and RhoGDI as nuclear and cytoplasmic markers, respectively (Fig. 6B, top panel). We then looked at the amount of phosphorylated ERKs in these fractions (Fig. 6B, bottom panel). Most interesting, in KBM5 the vast majority of activated ERKs (88%) was present in the cytoplasm, and barely 12% could be detected in the nucleus, in consonance with the high susceptibility of this cell line to STI571-induced apoptosis. On the other hand, in K562 cells, which displayed the highest resistance to STI571-induced apoptosis, about 36% of activated ERKs were nuclear. These results speak in favor of
ERK nuclear activity levels playing a major role in determining the ability of CML cells to counteract apoptosis evoked by STI571 treatment.

1xB/NFxBs Influence ERK2 Anti-apoptotic Effects Depending on the Stimulus and the Subcellular Location—Our final goal was to gain an initial insight into the mechanisms whereby activated ERK was affecting apoptosis in LMC cells. Thus, we investigated how compartmentalized ERK2 activation influenced several pathways known to exert anti-apoptotic effects in our cellular setting. Phosphorylation of the Bcl-2 family member BAD prevents its pro-apoptotic functions (52). Because BAD is phosphorylated by the Ras/ERK pathway among others (53) with the aid of phosphorylated BAD-specific antibodies, we investigated whether BAD phosphorylation was affected by site-restricted ERK2 activity. However, no major changes on phospho-BAD levels were detected among parental and E/M-, E/M LA-, and MEK E-expressing cell lines (data not shown). In the same fashion, we also analyzed the changes in the phosphorylation status of the transcription factor CREB, known to mediate in pro-survival mechanisms upon being phosphorylated by the ERK pathway (54). Similarly, we could not detect significant differences on CREB phosphorylation levels (data not shown).

Another well known mediator in anti-apoptotic processes in certain cell types is the transcription factor NFxB (55). As such, we tested how NFxB-dependent signaling pathways were activated as a result of compartmentalized ERK2 activity and whether they mediated in the anti-apoptotic effects of ERKs. To test if NFxB complexes were assembled as a response to site-restricted ERK2 activation under apoptogenic conditions, we performed EMSA with an NFxB-specific DNA probe and specific antibodies. As shown in Fig. 7A, left panel, NFxB complexes were present in E/M and E/M LA transfectants as well as in parental K562 cells under normal growing conditions, and exposing these cell lines to serum starvation did not induce major changes in the nature of the NFxB complexes. Conversely, treatment with STI571 resulted in the complete disappearance of the complexes from K562 parental cells and from E/M expressors, in which this drug induces apoptosis. On the other hand, NFxB complexes persisted in E/M LA and MEK E-expressing cells in which we had previously observed a resistance to STI571-induced apoptosis (Fig. 7A, right panel).

To substantiate further the involvement of NFxB in ERK anti-apoptotic response, we investigated if by inhibiting NFxB we could alter the protective effects that nuclear ERK2 activation provides against STI571-induced apoptosis. To this end, we utilized the inhibitor BAY11-7082, known to impede NFxB activation, by preventing 1xB phosphorylation (56). As we had shown before, in comparison to control K562 cells, in E/M LA transfectants the degree of apoptosis induced by STI571 was markedly reduced. Surprisingly, upon treatment with increasing concentrations of BAY11-7082, the susceptibility to STI571-induced apoptosis was reduced even further (Fig. 7B, left panel). More interesting, the effect exerted by BAY11-7082 was the opposite in untreated, proliferating E/M LA transfectants. Under these conditions, this drug exhibited a slight pro-apoptotic effect (Fig. 7B, right panel). It was of interest to determine whether the protective effect of BAY11-7082 against STI571-induced apoptosis observed in E/M LA transfectants guarded some relationship with the cellular location in which ERK2 was active. Thus we performed the same experiments in cells expressing E/M in which ERK2 is active in the cytoplasm. In this case, however, increasing concentrations of BAY11-7082 did not affect the apoptotic effect caused by STI571 (Fig. 7C, left panel). Finally, we explored if the inhibition of 1xB/NFxB could also potentiate ERK2 protective effects against apoptosis induced by other stimuli. We tested this in cells expressing E/M in which we have shown that cytoplasm-activated ERK2 protects against serum depletion-induced apoptosis. As shown in Fig. 7C, right panel, in E/M transfectants the degree of apoptosis caused after 5 days of serum starvation was unaltered by the incubation with the 1xB inhibitor BAY11-7082. In all, these
results suggest that the overall effects on apoptosis resulting from the interaction between ERKs and IκB/NFκB activities is strictly dependent on the cellular site where ERK2 activation ensues and the apoptotic stimulus is in action.

**DISCUSSION**

Although the relevance of the activation of ERKs in proliferative and differentiation processes in LMC cells is still a controversial issue currently under extensive scrutiny, a large body of data clearly support the involvement of ERKs in the generation of survival and/or anti-apoptotic signals in these cells (21). As such, it would be conceivable that those cells that exhibit higher ERK activity levels should display greater resistance against apoptotic stimuli. Indeed, this principle has been demonstrated previously in cells of diverse myeloid lineages (49). Here we demonstrate that it also applies to CML cell lines. However, it is noticeable that ERK activity levels and resistance to apoptosis do not follow a strict linear relationship. For example, the differences in ERK activity levels between K562 and KU812 cells are rather subtle, not even 2-fold, but K562 exhibits much greater resistance to apoptosis than KU812. One possible explanation for this observation would be that instead of a gradual increase in resistance to apoptosis as a function of augmenting ERK activity levels, there would be a threshold of ERK activation over which resistance to apoptosis is exacerbated. In this respect, it has been shown previously that decreasing the activity levels of ERKs by the use of MEK inhibitors, such as PD98059 and UO126, greatly augments K562 sensitivity to apoptogenic stimuli (49, 50). Here we show that this is also the case for other CML cell lines such as KU812 and KBM5, in which treatment with UO126 makes them more
sensible to serum depletion-induced apoptosis. It should be noticed that in all of the aforementioned cases, the effects of the MEK inhibitors are somewhat modest. This could be explained by the known fact that the ability of these inhibitors to interfere with MEK activation is greatly diminished under conditions in which MEK is already stimulated (57), as is the case in our models using proliferating cells. Thus, it is very likely that the effects on apoptosis of down-regulating the ERK pathway may be underestimated when interpreting the results of experiments using MEK inhibitors.

In any case, precaution must be exercised when attempting to correlate the activity levels of ERKs and apoptosis, because a large body of evidence indicates that rather than the intensity of the isolated ERK signal, it is the balance among the intensities of the ERK anti-apoptotic and the c-Jun NH$_2$-terminal kinase/p38 pro-apoptotic signals that will ultimately determine the susceptibility of a cell to undergo apoptosis (1).

In light of the unquestionable role of ERKs in cell survival signals, we have placed an emphasis in investigating the consequences of compartmentalizing ERK activity in the apoptotic response of CML cell lines. It is well known that once activated ERKs disperse throughout the cell and phosphorylate multiple effectors that are distributed in different subcellular localizations (1, 58, 59). Indeed, ERKs can regulate some biochemical processes in a localization-dependent fashion. For instance, membrane-proximal but not cytoplasmic ERK signaling is required for activation of M-calpain by epidermal growth factor (60). ERKs themselves are subject to regulatory processes strictly dependent on cellular localization, as we have recently demonstrated (40). Therefore, it is likely that the relative contributions of these location-confined ERK signal components to a given biological output are markedly distinct. This notion is supported by evidence demonstrating that ERK activation restricted to the nucleus is sufficient to induce differentiation in PC12 cells and transformation of NIH3T3 fibroblasts (39). Accordingly, blocking ERK nuclear translocation prevents c-Fos transcription and proliferation (61, 62). Here we demonstrate that the anti-apoptotic effects of ERK can also be exerted in a site-dependent fashion, depending on the impinging apoptotic stimulus. As such the protective effect against growth factor depletion is carried out by cytoplasmic ERK, whereas the anti-apoptotic response against STI571 takes place in the nucleus. In line with our data, in D2 myeloid cells phosphorylated ERK accumulates in the cytoplasm of phorbol ester-induced
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pro-apoptotic cells (63), and recent results indicate that PEA-Bcr-Abl activates NF-kB by a mechanism independent of IkB phosphorylation. This would be consistent with recent data indicating that IκB constantly shuttles in and out of the nucleus (73, 74).

Nevertheless, our data showing that, irrespective of ERK2 being active in the nucleus, BAY11-7082 exerts completely opposite effects, depending on whether Bcr-Abl is active or not, poses a conceptual challenge worth investigating. Overall, even though the downstream mediators have yet to be firmly identified, our results demonstrate for the first time that the protective response of ERKs against defined apoptogenic stimuli is dependent on the cellular localization where ERK activation occurs.

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