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MEK kinase 1 mediates the antiapoptotic effect of the *Bcr-Abl* oncogene through NF-κB activation

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Bcr-Abl tyrosine kinase, a chimeric oncoprotein responsible for chronic myelogenous leukemia, constitutively activates several signal transduction pathways that stimulate cell proliferation and prevent apoptosis in hematopoietic cells. The antiapoptotic function of Bcr-Abl is necessary for hematopoietic transformation, and also contributes to leukemogenesis. Herein, we show for the first time that cell transformation induced by Bcr-Abl leads to increased expression and kinase activity of MEK kinase 1 (MEKK1), which acts upstream of the c-Jun N-terminal kinase (JNK), extracellular signal regulated kinase (ERK) and NF-κB signaling pathways. Inhibition of MEKK1 activity using a dominant-negative MEKK1 mutant (MEKK1km) diminished the ability of Bcr-Abl to protect cells from genotoxin-induced apoptosis, but had no effect on the proliferation of Bcr-Abl-transformed cells. Expression of MEKK1km also reduced NF-κB activation, and inhibited antiapoptotic c-IAP1 and c-IAP2 mRNA expression in response to the genotoxin. By contrast, neither JNK nor ERK activation was affected. These results indicate that MEKK1 is a downstream target of Bcr-Abl, and that the antiapoptotic effect of Bcr-Abl in chronic myelogenous leukemia cells is mediated via the MEKK1-NF-κB pathway.
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Introduction

Chronic myelogenous leukemia (CML) is a neoplasm arising from myeloid progenitor cells expressing the 210-kDa form of Bcr-Abl, a chimeric oncoprotein generated by the reciprocal translocation t(9;22) (Deininger *et al*., 2000). This oncoprotein is responsible for the phosphorylation, activation and dysregulation of various intracellular signaling proteins that regulate the proliferation and survival of progenitor cells. For instance, introduction of a *Bcr-Abl* fusion gene into growth factor-dependent cell lines rapidly renders them growth factor-independent (Daley and Baltimore, 1988), while expression of Bcr-Abl protein has been implicated in the resistance of CML to apoptosis-inducing antileukemia drugs (Skorski, 2002). In addition, by suppressing mitochondrial release of cytochrome *c* and blocking the cytosolic pathway leading to activation of caspase-3, Bcr-Abl blocks the apoptosis induced by a variety of stimuli (Amarante-Mendes *et al*., 1998; Dubrez *et al*., 1998). Bcr-Abl can also affect *c-jun* expression, JNK activation and the NF-κB pathway, which may contribute to apoptosis suppression, transformation and tumorigenesis (Raitano *et al*., 1995; Burgess *et al*., 1998; Reuther *et al*., 1998). Still, the precise mechanisms underlying Bcr-Abl-induced leukemogenesis remain unclear.

MEKK1 is a 196-kDa serine–threonine kinase activated in response to a variety of stimuli, including epidermal growth factor (EGF), lysophosphatidic acid, osmotic stress and anticancer drugs (Fanger *et al*., 1997; Yujiri *et al*., 1998, 1999). Upon activation, MEKK1 participates in the regulation of JNK and ERK pathways, and is involved in the activation of NF-κB (Lee *et al*., 1997). Recently, c-Abl was shown to associate with MEKK1 and to act upstream of the MEKK1-dependent activation of JNK stimulated by genotoxic stress (Kharbanda *et al*., 2000). However, it is not known whether MEKK1 is involved in the Bcr-Abl-induced transformation of hematopoietic cells. The experiments described herein demonstrate that MEKK1 is a downstream target of Bcr-Abl, mediating Bcr-Abl's antiapoptotic effect via NF-κB in a manner that may involve the NF-κB-regulated genes c-IAP1 and c-IAP2.

Results

Bcr-Abl increases MEKK1 expression and enhances its kinase activity

We began investigating the relationship between Bcr-Abl and MEKK1 by evaluating MEKK1 expression in p210 Bcr-Abl-transformed Ba/F3 and 32D cells. We found that expression of MEKK1 protein was

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constitutively enhanced in both cell types, as compared to the respective parental cells. In addition, an upward shift in the MEKK1 band, reflecting the protein's phosphorylation/activation (Widmann *et al*., 1998a, b), was enhanced in Bcr-Abl-transformed cells (Figure 1a). Furthermore, *in vitro* kinase assays (Lu *et al*., 2002) confirmed that the activity of endogenous MEKK1, even adjusted for the protein level, was significantly enhanced in both Bcr-Abl-transformed Ba/F3 and 32D cells (Figure 1b).

Bcr-Abl exhibits constitutive tyrosine kinase activity, which is essential for its transforming activity in CML (Deininger *et al*., 2000). To investigate the relationship between Bcr-Abl tyrosine kinase activity and MEKK1 expression and kinase activity more precisely, we examined the effects of imatinib mesilate (STI571), a selective inhibitor of Bcr-Abl tyrosine kinase activity (Druker, 2002). CrkL is one of the most prominently tyrosine-phosphorylated proteins in Bcr-Abl-transformed cells (Oda *et al*., 1994), and is a good indicator of Bcr-Abl tyrosine kinase activity (Gorre *et al*., 2001). Addition of STI571 to Bcr-Abl-transformed Ba/F3 cells reduced the level of phosphorylated CrkL, as indicated by a reduction in the upward shift on gels (Figure 1c). Moreover, there was a corresponding time-dependent decline in the expression of MEKK1 protein (Figure 1c) and in MEKK1 enzymatic activity (Figure 1d). All of these effects appear to be MEKK1-specific, as the expression of MEKK2, another MEKK family protein, was never affected (Figure 1a and c).

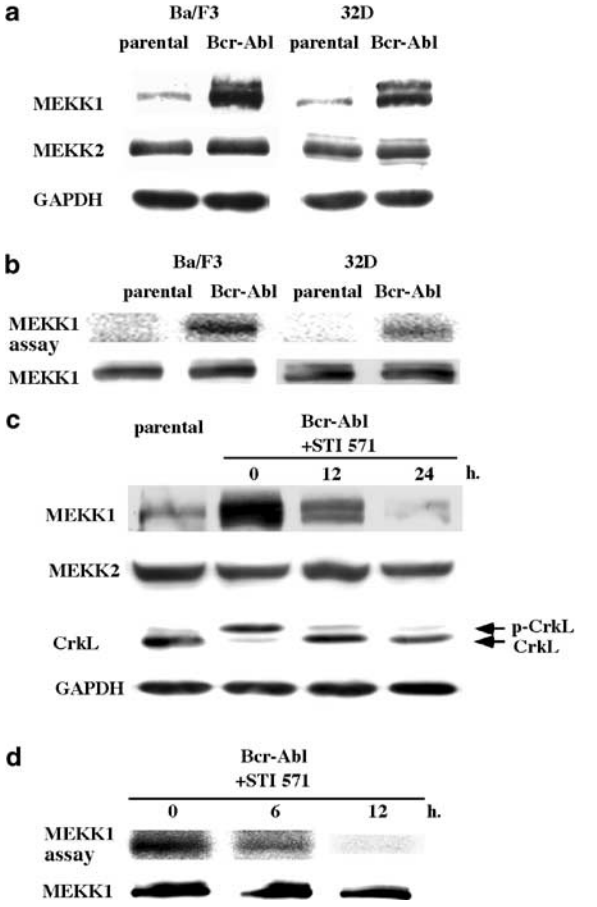

Figure 1 Bcr-Abl enhances the expression and kinase activity of MEKK1. (**a**) Increased expression of MEKK1 in Ba/F3 and 32D cells expressing p210 Bcr-Abl. Parental cells (parental) and p210 Bcr-Abl transfectants (Bcr-Abl) were seeded to a density of 2×10^5 cells/ml. After 12 h, the cells were harvested, and cell lysates were immunoblotted for MEKK1 and MEKK2. (**b**) Enhanced MEKK1 kinase activity in p210 Bcr-Abl-transformed Ba/F3 and 32D cells. Cell lysates containing equal amounts of MEKK1 were prepared, after which endogenous MEKK1 was immunoprecipitated and incubated with [γ-^{32}P] ATP, or immunoblotted with MEKK1 Ab. (**c**) An Abl tyrosine kinase inhibitor reduces the expression of MEKK1. Bcr-Abl-transformed Ba/F3 cells were seeded to a density of 2×10^5 cells/ml. After 12 h, 5 μM STI571 was added and the cells were incubated for the indicated times. The cells were then harvested, and cell lysates were immunoblotted for MEKK1, MEKK2 and CrkL. Levels of GAPDH indicated that equivalent amounts of protein had been loaded into each lane (**a**, **c**). (**d**) An Abl tyrosine kinase inhibitor reduces MEKK1 kinase activity. Bcr-Abl-transformed Ba/F3 cells were cultured as in panel (**c**), and incubated with 5 μM STI571 for the indicated times. Cell lysates of equivalent amounts of MEKK1 protein were then prepared, and endogenous MEKK1 was immunoprecipitated and incubated with [γ-^{32}P]ATP in an *in vitro* kinase assay, or immunoblotted with MEKK1 Ab

A dominant-negative MEKK1 mutant (MEKK1km) inhibits the antiapoptotic effect of Bcr-Abl

We next investigated the function of MEKK1 by stably expressing a dominant-negative MEKK1km in both parental and Bcr-Abl-transformed Ba/F3 cells using retroviral expression vectors. Expression of MEKK1km did not affect the proliferation rate of either cell type in the presence or absence of IL-3 (Figure 2a, left and right panels, respectively). Similar results were observed in 32D cells (data not shown). Likewise, the level of CrkL phosphorylation, reflecting Bcr-Abl tyrosine kinase activity, was unaffected by the expression of MEKK1km (Figure 2b).

On the other hand, apoptosis induced by the genotoxin etoposide was diminished in MEKK1km-expressing Ba/F3 cells (Figure 3a), which is consistent with earlier observations in HEK293 cells (Widmann *et al*., 1998a; Gibson *et al*., 1999). Notably, expression of MEKK1km had the opposite effect in Bcr-Abl-transformed cells – that is, the incidence of etoposide-induced apoptosis was significantly higher in cells expressing Bcr-Abl plus MEKK1km (Bcr-Abl + MEKK1km) than in cells expressing Bcr-Abl alone (Bcr-Abl) in the absence or presence of IL-3 (Figure 3b). Expression of MEKK2km with Bcr-Abl did not augment the incidence of etoposide-induced apoptosis, further confirming that the activity of Bcr-Abl is MEKK1-specific.

The antiapoptotic effect of Bcr-Abl requires NF-κB activation

Expression of *Bcr-Abl* reportedly leads to activation of a variety of downstream signaling molecules (Deininger *et al*., 2000). To investigate the signaling mediating the antiapoptotic effect of Bcr-Abl via MEKK1, we first

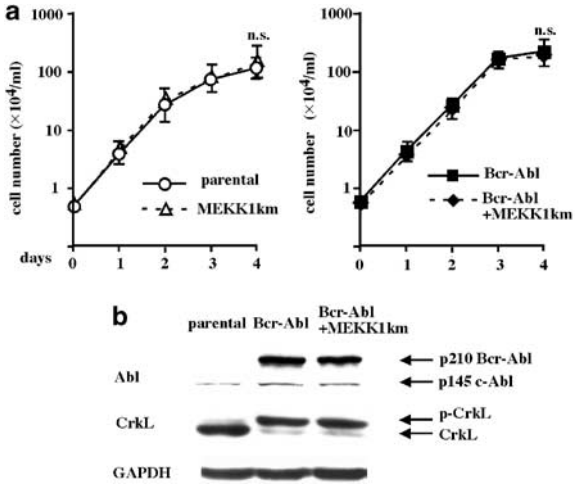

Figure 2 A dominant-negative MEKK1km does not affect proliferation or Bcr-Abl tyrosine kinase activity in Bcr-Abl-transformed Ba/F3 cells. (**a**) Ba/F3 parental cells (○) and cells stably transfected with a retrovirus harboring a dominant-negative MEKK1km (MEKK1km, (△)), p210 Bcr-Abl (Bcr-Abl, (■)) or p210 Bcr-Abl plus MEKK1km (Bcr-Abl + MEKK1km, (♦)) were seeded to a density of 7×10^3 cells/ml in the presence (left panel) or absence (right panel) of IL-3, and harvested on day 1, 2, 3 or 4. Live cells were counted using trypan blue staining. The data are means ± s.d. from three independent experiments; n.s., not significant (Student's *t*-test). (**b**) Cell lysates from parental Ba/F3 cells and Bcr-Abl and Bcr-Abl + MEKK1km transfectants were analysed by immunoblotting with c-Abl, CrkL and GAPDH Abs

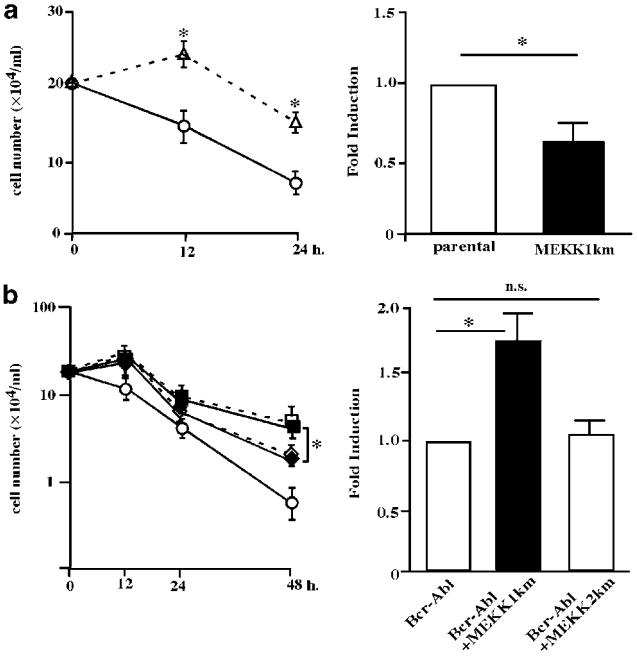

Figure 3 MEKK1 mediates the antiapoptotic effect of Bcr-Abl. (**a**) MEKK1km suppresses etoposide-induced apoptosis in parental Ba/F3 cells. Cells were grown to a density of 2×10^5 cells/ml in the presence of IL-3, and then exposed to 50 μM etoposide. Numbers of viable parental Ba/F3 cells (○) and MEKK1km transfectants (△) were determined using trypan blue staining (left panel); relative numbers of apoptotic cells were determined by propidium iodide and annexin-V staining (right panel). (**b**) MEKK1km enhances etoposide-induced apoptosis in Bcr-Abl-transformed Ba/F3 cells. Cells were grown to a density of 2×10^5 cells/ml in the presence or absence of IL-3, and then exposed to 50 μM etoposide for the indicated time periods. Numbers of viable cells (parental Ba/F3 cells with IL-3 (○), Bcr-Abl without IL-3 (■), with IL-3 (□), Bcr-Abl + MEKK1km without IL-3 (♦), with IL-3 (◇) (left panel) and relative numbers of apoptotic cells (right panel) were determined as in (**a**). The data in both panels are means ± s.d. from three independent experiments; $^*P < 0.05$ (Student's *t*-test)

analysed the activation of the MAPK pathways in Bcr-Abl and Bcr-Abl + MEKK1km cells using several anti-phospho-MAPK antibodies (Abs) (Figure 4a). Levels of endogenous JNK and ERK were unaffected by exposure of cells to etoposide (data not shown). Activations of JNK and ERK peaked after 12 h of exposure to etoposide, but no significant difference was detected in the degree of activation between Bcr-Abl and Bcr-Abl + MEKK1km cells. Activation of p38 was not detected at any time, though levels of endogeneous p38 were equal in the two cell types. Bcr-Abl also reportedly activates phosphatidyl inositol 3-kinase (PI3K)-Akt as well as signal transducers and activators of transcription (Stat) (Ilaria and Van Etten, 1996; Shuai *et al*., 1996; Skorski *et al*., 1997). When we compared levels of Akt and Stat5 activation using phosphorylation-specific Abs in Bcr-Abl and Bcr-Abl + MEKK1km cells, we found that Akt was not activated in response to etoposide treatment in either cell type, whereas Stat5 was constitutively activated, irrespective of etoposide stimulation (Figure 4b).

On the other hand, NF-κB was previously implicated in the antiapoptotic action of Bcr-Abl and is known to play an important role in cell transformation by Bcr-Abl (Reuther *et al*., 1998). Using electrophoretic mobility shift assays (EMSAs), we found that activation of NF-κB peaked after 6 h of etoposide exposure in both Bcr-Abl and Bcr-Abl + MEKK1km cells (Figure 5). However, expression of MEKK1km significantly reduced the level of NF-κB activation at all times. It thus appears that, among the molecules tested, only NF-κB is involved in the cell survival mediated via the Bcr-Abl-MEKK1 pathway.

MEKK1km inhibits genotoxin-induced expression of c-IAP1 and c-IAP2 mRNA

In our final experiment, we investigated several anti- or proapoptotic proteins known to be induced by NF-κB activation as downstream targets of the Bcr-Abl-MEKK1 pathway. We found expression of the apoptosis-related proteins Bcl-2, Bcl-xL, Mcl-1, Bad and Bid to be unaffected by etoposide treatment in either Bcr-Abl or Bcr-Abl + MEKK1km cells (Figure 6). By contrast, whereas expressions of c-IAP1 and c-IAP2 mRNA were similar in the two cell types prior to etoposide treatment, after treatment their expressions were significantly lower in Bcr-Abl + MEKK1km cells (Figure 7). This suggests that NF-κB-induced mobilization of c-IAP1 and c-IAP2, which potently inhibit caspases (Salvesen and

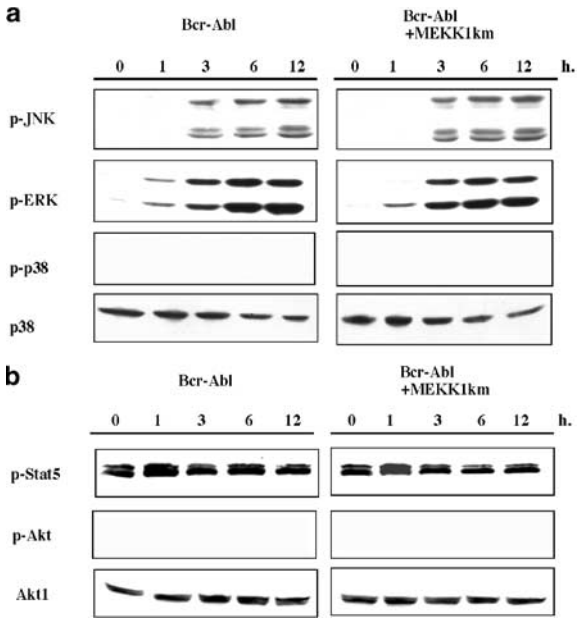

Figure 4 MEKK1km does not affect etoposide-induced MAPK, Stat or Akt activation. (**a**) and (**b**), Cells expressing Bcr-Abl or Bcr-Abl + MEKK1km were grown to a density of 2×10^5 cells/ml in the absence of IL-3, exposed to 50 μM etoposide and harvested at the indicated time points. Cell lysates were analysed by immunoblotting with Abs against p-JNK, p-ERK, p-p38 or p38 (**a**) or against p-Stat5, p-Akt or Akt1 (**b**). Equivalent amounts of protein were loaded into each lane. The immunoblotting shown is representative of three independent experiments yielding similar results

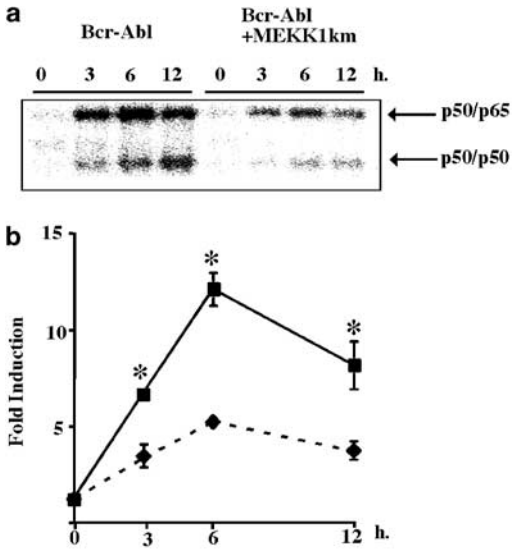

Figure 5 MEKK1km inhibits etoposide-induced NF-κB activation in Bcr-Abl-transformed cells. (**a**) EMSA using nuclear extracts prepared from cells expressing Bcr-Abl (■) or Bcr-Abl + MEKK1km (♦). The cells were exposed to etoposide as in Figure 4. The autoradiogram shown is representative of three independent experiments yielding similar results. (**b**) The intensity of the p50/p65 bands was quantified using FLA 2000. The values before treatment of etoposide are defined as 1.0 for each cell line. The data in both panels are means ± s.d. from three independent experiments; *$P < 0.05$ (Student's *t*-test)

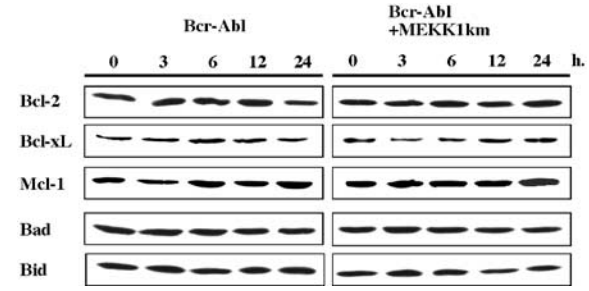

Figure 6 MEKK1km does not affect levels of Bcl-2 family proteins in etoposide-treated, Bcr-Abl-transformed cells. Cells expressing Bcr-Abl and Bcr-Abl + MEKK1km were treated with etoposide as in Figure 4, after which cell lysates were analysed by immunoblotting with Bcl-2, Bcl-xL, Mcl-1, Bad or Bid Ab. The immunoblotting shown is representative of three independent experiments yielding similar results

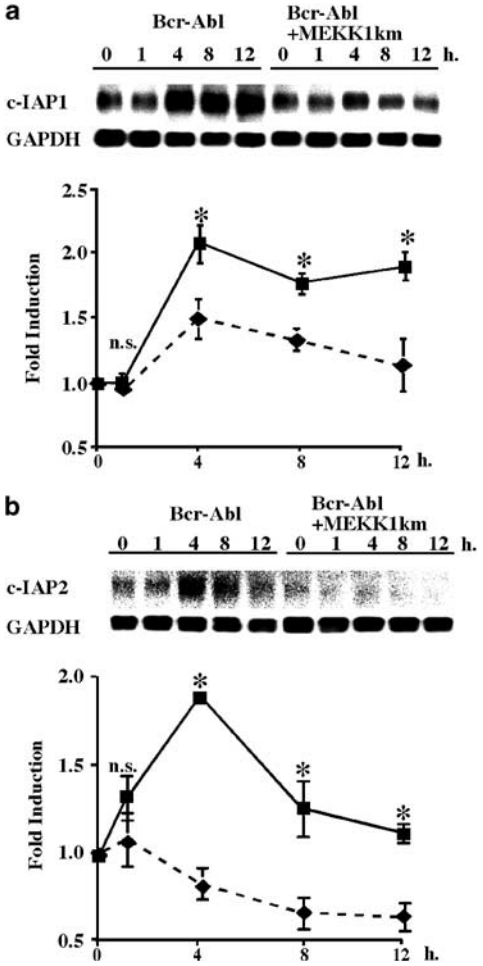

Figure 7 MEKK1km inhibits expressions of c-IAP1 and c-IAP2 mRNA in Bcr-Abl-transformed cells. Cells expressing Bcr-Abl (■) or Bcr-Abl + MEKK1km (♦) were treated as in Figure 4, and then harvested at the indicated times. Total RNAs were resolved on formaldehyde gels and subjected to Northern blot analysis. Murine c-IAP1 and c-IAP2 and GAPDH cDNAs were used as probes. The autoradiograms shown are representative of three independent experiments yielding similar results. The values before treatment of etoposide are defined as 1.0 for each cell line. The symbols are means ± s.d.; *$P < 0.05$; n.s., not significant (Student's *t*-test)

Duckett, 2002), plays a key role in the antiapoptotic signaling of Bcr-Abl via MEKK1.

Discussion

We have shown that MEKK1 is a downstream target of Bcr-Abl signaling and contributes to its antiapoptotic effect. The phenotype of Bcr-Abl-transformed cells is characterized by growth factor-independent proliferation and reduced susceptibility to apoptosis induced by various stimuli, including genotoxins such as etoposide, which together contribute to leukemogenesis (Cortez *et al*., 1995). Moreover, the antiapoptotic effect of Bcr-Abl, in hematopoietic cell lines at least, is separable from its ability to induce growth factor independence (Kabarowski *et al*., 1994). Our data suggest that MEKK1 contributes to Bcr-Abl's antiapoptotic effect, but is not involved in growth factor-independent cell growth.

Our experiments further showed MEKK1 expression and its kinase activity to be increased in Bcr-Abl-transformed cells, and that introduction of MEKK1km renders these cells more susceptible to etoposide-induced apoptosis. We therefore suggest that Bcr-Abl transmits its prosurvival signal via MEKK1. Interestingly, MEKK1 is also known to transduce proapoptotic signals. For example, many apoptotic stimuli, including genotoxins, activate MEKK1 (Widmann *et al*., 1998b; Gibson *et al*., 1999), and overexpression of MEKK1 leads to caspase activation and apoptosis (Widmann *et al*., 1998a). Moreover, expression of MEKK1km inhibits genotoxin and anoikis, the loss of integrin-mediated contacts with extracellular matrix-induced apoptosis (Cardone *et al*., 1997; Widmann *et al*., 1998a). It also inhibits etoposide-induced NF-κB activation, and diminishes the expression of death receptors 4 and 5 in HEK293 cells (Gibson *et al*., 2000). In the present study, MEKK1km inhibited etoposide-induced apoptosis in parental Ba/F3 cells, but exhibited the opposite effect in Bcr-Abl-transformed cells. Similarly, a dominant-negative MEKK1km strongly suppressed the survival of pancreatic cancer cells, but not of nonpancreatic cancer cells (Hirano *et al*., 2002). The specific function of MEKK1 during stress-induced apoptosis thus appears to be context-specific.

IAP family proteins are characterized by a novel 70-amino-acid domain, termed the baculoviral IAP repeat (BIR), a name that derives from the original discovery of these apoptosis suppressors in the genomes of baculoviruses (Crook *et al*., 1993). Regulated by NF-κB, they appear to function as 'guardians' of the cell death machinery by directly binding to and neutralizing caspases (Chu *et al*., 1997; Salvesen and Duckett, 2002). Our finding that MEKK1km inhibited etoposide-induced NF-κB activation and c-IAP1 and c-IAP2 mRNA expression suggests that MEKK1 might protect CML cells from apoptosis via induction of IAP family proteins through NF-κB activation.

Bcl-2 family proteins are also potential mediators of the antiapoptotic effect of Bcr-Abl; indeed, they were previously shown to play a key role in the antiapoptotic response to genotoxin (Makin and Dive, 2001). However, after exposing cells to etoposide, we detected no difference in the protein levels of Bcl-2, Bcl-xL, Mcl-1, Bad or Bid in either Bcr-Abl or Bcr-Abl + MEKK1km cells. These data suggested that MEKK1 does not, at least, contribute to Bcl-2 family protein levels after treatment of Bcr-Abl-transformed cells with etoposide. It might be still possible that these Bcl-2 family proteins exert antiapoptotic effects via some modification such as Bcl-xL deamidation (Deverman *et al*., 2002), rather than via changes in protein levels.

Akt has been shown to be constitutively activated in Bcr-Abl cells (Skorski *et al*., 1997). We detected no Akt activation (phosphorylation) in our Bcr-Abl cells, regardless of etoposide treatment, despite repeated attempts. It might be cell type specific, and/or partly dependent on our assay using immunoblotting with anti-phospho-Akt (Ser473) Ab, which is not an *in vitro* kinase assay.

Bcr-Abl causes the adhesion defects and cytoskeletal abnormalities characteristic of CML cells by interacting with cell membrane proteins and proteins involved in cytoskeletal organization – for example, paxillin, actin, talin, vinculin and focal adhesion kinase (FAK) (Salgia *et al*., 1997). The observations that MEKK1, which is upregulated in Bcr-Abl-transformed cells, is involved in cell migration and interacts with FAK, α-actinin and paxillin (Christerson *et al*., 1999; Yujiri *et al*., 2000, 2003) suggest that, in addition to mediating a prosurvival signal, constitutively activated MEKK1 may disrupt cell adhesion and migration in Bcr-Abl-transformed cells.

Finally, MEKK1km enhances apoptosis in etoposide-treated Bcr-Abl-transformed cells. This observation indicates that MEKK1 may be a useful therapeutic target in the treatment of CML.

Materials and methods

Cell culture

Ba/F3, an IL-3-dependent murine pro-B cell line, and 32D, an IL-3-dependent murine myeloid precursor cell line, were obtained from the Riken Cell Bank (Saitama, Japan). These cells were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen), 10% fetal bovine serum (Equitech-Bio, Inc. Ingram, TX, USA) and 1 ng/ml recombinant murine IL-3 (Kirin Brewery, Tokyo, Japan). Bcr-Abl-expressing Ba/F3 and 32D cells were selected by withdrawal of IL-3. Dominant-negative MEKK1- and MEKK2-expressing cells were selected by adding 1 and 0.6 μg/ml of puromycin to the culture medium for Ba/F3 and 32D cells, respectively. All of these cell lines were used as polyclonal cell mixtures.

Materials

Anti-MEKK2, anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), anti-CrkL, anti-JNK1, anti-ERK2, anti-Akt1

and anti-p38 Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Bcl-2, anti-Bcl-xL, anti-Mcl-1, anti-Bad and anti-Bid Abs were from BD Transduction (Lexington, KY, USA). Anti-c-Abl Ab was from Oncogene Research (Cambridge, MA, USA). Anti-phospho-Stat5(Tyr 694) Ab was from Cell Signaling Technology, Inc. (Beverly, MA, USA). Anti-NH3-terminal-specific MEKK1 Ab was described elsewhere (Fanger *et al*., 1997). The retroviral vectors pMX and pMY were prepared as described previously (Onishi *et al*., 1996). p210-Bcr-Abl cDNA was a gift from Dr Owen N Witte (UCLA). Dominant-negative MEKK1 and MEKK2 mutants (MEKK1km and MEKK2km, respectively), created by substitution of methionine for lysine in the ATP-binding domain, were kindly provided by Dr Gary L Johnson (Colorado University). Ba/F3 and 32D cells were stably transformed with pMY-p210-Bcr-Abl, pMX-MEKK1km or pMX-MEKK2km, after which they were selected by IL-3 deprivation (Bcr-Abl) or incubation with 1 and 0.6 μg/ml of puromycin (MEKK1km and MEKK2km) to the culture medium for Ba/F3 and 32D cells, respectively. STI571 was kindly provided by Novartis (Basel, Switzerland).

Stock solutions of STI571 (10 mM) and etoposide (100 mM) (Sigma) were prepared in dimethylsulfoxide, and stored at -20°C. Appropriate drug concentrations were made by dilution with fresh medium immediately before each experiment. The final concentration of dimethylsulfoxide in the media was less than 0.1%, and had no effect on cell growth in the present study.

Immunoblotting

Cells were lysed in buffer containing 20 mM Tris-HCl (pH 7.6), 0.5% NP40, 250 mM sodium chloride, 3 mM EDTA, 3 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 2 mM sodium orthovanadate, 20 μg/ml aprotinin, 1 mM dithiothreitol and 5 μg/ml leupeptin. The resultant lysates were centrifuged at 14 000 *g* for 10 min at 4°C, after which the supernatant was collected, and the samples were subjected to SDS–PAGE. The resolved proteins were transferred to nitrocellulose membranes. Protein concentrations in the lysates were measured using Bradford assays with bovine serum albumin as a standard.

In vitro *MEKK1 kinase assay*

Cells were grown to a density of 2×10^5 cells/ml, after which they were lysed and centrifuged as described above. Cell lysates were immunoblotted with anti-MEKK1 Ab, and relative MEKK1 expression levels were determined with NIH Image 1.62. Aliquots of cell lysate containing equal amounts of MEKK1 protein were incubated with anti-MEKK1 Ab for 2 h at 4°C, and then with protein-G sepharose (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for one additional hour. The sepharose beads were first washed in lysis buffer, then twice in kinase buffer (20 mM HEPES (pH 7.5), 10 mM β-glycerophosphate, 10 mM *p*-nitrophenyl phosphate, 10 mM magnesium chloride, 1 mM dithiothreitol and 50 μM sodium orthovanadate). The resultant immunoprecipitates were incubated for 20 min at 30°C in kinase buffer containing 10 μCi [γ-^{32}P]ATP (ICN Biologicals). The kinase reaction was stopped by addition of SDS sample buffer, after which the proteins were separated by SDS–PAGE and visualized with autoradiography.

Analysis of kinase activity

To assess the levels of JNK, ERK, p38 and Akt activation, cell lysates were immunoblotted with anti-phospho-JNK, MAPK, p38 or Akt(Ser 473) Ab (Cell Signaling Technology, Inc.), respectively. NIH Image 1.62 was then used to determine the relative levels of the activated enzymes.

Electrophoretic mobility shift assay (EMSA)

Cells were treated with 50 μM etoposide for the indicated times, washed twice with PBS and lysed for 10 min at 4°C in buffer containing 10 mM HEPES (pH 7.8), 10 mM sodium chloride, 0.5 mM EDTA, 1.5 mM magnesium chloride, 2 mM dithiothreitol, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, 10 μg/ml leupeptin and 0.5 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride. NP40 was added to the resultant lysate to a final concentration of 1.2%. Nuclear pellets, obtained by centrifugation at 10 000 *g* for 4 min at 4°C, were incubated for an additional 30 min at 4°C in buffer containing 10 mM HEPES (pH 7.8), 420 mM sodium chloride, 0.5 mM EDTA, 1.5 mM magnesium chloride, 5 mM dithiothreitol, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, 0.5 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride and 10% glycerol, after which nuclear protein extracts were prepared by centrifugation at 14 000 *g* for 10 min at 4°C. Aliquots of nuclear extract (8 μg protein) were preincubated for 15 min at room temperature with 1 μg of poly(dI-dC) in binding buffer (10 mM Tris HCl (pH 7.5), 100 mM potassium chloride, 50% glycerol, 1 mM dithiothreitol, 0.5 mM EDTA). A ^{32}P-labeled DNA probe for the NF-κB-binding site (Yujiri *et al*., 2000) was then added, and the incubation was continued for an additional 15 min. The DNA–protein complexes were then separated on a 6% polyacrylamide gel and exposed for autoradiography. NF-κB DNA-binding activity was quantitated using FLA2000 (Fuji Film, Tokyo, Japan).

Cell viability and apoptosis assays

Cell viability was assessed by trypan blue staining. Apoptosis was measured using propidium iodide and annexin-V (TACS AnnexinV-FITC, R&D systems), according to the manufacturer's protocol. The binding of FITC-conjugated annexin-V and propidium iodide was assessed by fluorescence-activated cell separation (Coulter EPICS XL, Miami, FL, USA). To induce apoptosis, cells were grown to a density of 2×10^5 cells/ml, and then exposed to 50 μM etoposide for the indicated time periods.

Northern blotting

Total RNA was extracted from cells using ISOGEN (Nippongene, Japan), after which 15 μg samples were denatured in formaldehyde/formamide, resolved by electrophoresis, and transferred to Hybond membranes (Amersham Pharmacia Biotech.). The probes used were murine c-IAP1 and c-IAP2 cDNA fragments and the full-length GAPDH cDNA. The PCR primers used to construct the murine c-IAP1 probe were 5′-GAT GAA GAG TGC TGA CAC CT-3′ and 5′-GCA AAG CTC AAA GGC ATG GT-3′ (Genbank Accession U88908); for c-IAP2, the primers were 5′-GAC AAA ACT GTC TCC CAG AG-3′ and 5′-CTT AGA TGG AGA CTG CAG AC-3′ (GenBank Accession U88909).

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