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Interleukin 3-dependent activation of DREAM is involved in transcriptional silencing of the apoptotic *hrk* gene in hematopoietic progenitor cells

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The apoptotic protein Hrk is expressed in hematopoietic progenitors after growth factor deprivation. Here we identify a silencer sequence in the 3′ untranslated region of the *hrk* gene that binds to the transcriptional repressor DREAM in interleukin-3 (IL-3)-dependent hematopoietic progenitor cells, and abrogates the expression of reporter genes when located downstream of the open reading frame. In addition, the binding of DREAM to the *hrk* gene is reduced or eliminated when cells are cultured in the absence of IL-3 or treated with a calcium ionophore or a phosphatidylinositol 3-kinase-specific inhibitor, suggesting that both calcium mobilization and phosphorylation can regulate the transcriptional activity of DREAM. Furthermore, we have shown that DREAM is phosphorylated by a phosphatidylinositol 3-kinase-dependent, but Akt-independent pathway. In all cases, loss of the DREAM–DNA binding complex was correlated with increased levels of Hrk and apoptosis. These data suggest that IL-3 may trigger the activation of DREAM through different signaling pathways, which in turn binds to a silencer sequence in the *hrk* gene and blocks transcription, avoiding inappropriate cell death in hematopoietic progenitors.

Keywords: apoptosis/DREAM/*hrk*/transcription

Introduction

Apoptotic cell death plays a critical role in the development of hematopoietic progenitors. It is well documented that both growth factors and stromal cell interactions maintain survival of progenitor cells within the bone marrow, and that the absence or reduction of these survival signals induces cells to undergo apoptosis (Williams *et al*., 1990; Yu *et al*., 1993).

In hematopoietic progenitors, several mechanisms have been proposed to explain how growth factors regulate survival through Bcl-2 family members. Several hematopoietins, including interleukin-3 (IL-3) and erythropoietin, have been shown to maintain the protein levels of Bcl-2, Bcl-x_L, Mcl-1, Bax, Bad and Bak in both cell lines and primary myeloid progenitors (Packham *et al*., 1998; Sanz *et al*., 2000). In addition, growth factors can promote survival through post-translational modifications such as phosphorylation of Bad via activation of Akt kinase (del Peso *et al*., 1997). We have shown previously that the apoptotic protein Hrk, a recently described BH3-only member of the Bcl-2 family (Imaizumi *et al*., 1997; Inohara *et al*., 1997), is tightly controlled at the transcriptional level in hematopoietic progenitors (Sanz *et al*., 2000). The expression of Hrk is undetectable in cells cultured with growth factors, but is specifically upregulated upon growth factor deprivation, and this pattern of expression correlates with induction of apoptosis. Furthermore, ectopic expression of Hrk induced cell death of hematopoietic progenitors cultured with IL-3, indicating that the presence of this protein is sufficient to induce apoptosis.

Identifying the factors and signaling pathways that regulate the levels of Bcl-2 family members is important in understanding better the response of cells to apoptotic stimuli such as chemotherapeutic drugs. Some of the key transcriptional activators of anti-apoptotic members have been identified (Lee *et al*., 1999; Tamatani *et al*., 1999; Lord *et al*., 2000). For example, it has been described that Stats and NFκB factors transactivate the *bcl-x* gene in a variety of cell systems (Socolovsky *et al*., 1999; Chen *et al*., 2000), and that anti-apoptotic NFκB signaling can be activated by the phosphatidylinositol 3 (PI 3)-kinase/Akt pathway (Romashkova and Makarov, 1999). In contrast, few data exist regarding negative regulatory factors for apoptotic members of the Bcl-2 family, although transcriptional repression of strong apoptosis inducers would be a safeguard mechanism to avoid inappropriate cell death in normal hematopoietic progenitors. We report here the identification of a downstream regulatory element (DRE) sequence in the 3′ untranslated region (UTR) of the *hrk* gene. In IL-3-dependent hematopoietic progenitor cell lines, this sequence binds to a calcium-binding protein, DREAM, which functions as a transcriptional repressor (Carrion *et al*., 1999). Furthermore, the DREAM-binding sequence (DRE-hrk) abrogates the expression of reporter genes when located downstream of the open reading frame. In addition, we show that the activity of DREAM can be regulated by calcium mobilization and also by direct phosphorylation through a PI 3-kinase-dependent, but Akt-independent pathway, which may allow this repressor to bind DNA and inhibit transcription of hrk in the presence of IL-3.

Results

The hrk gene contains a DRE that binds to the transcriptional repressor DREAM in hematopoietic progenitors

We have shown previously that Hrk is not expressed in viable hematopoietic progenitors, but it is specifically induced at the mRNA and protein level after growth factor

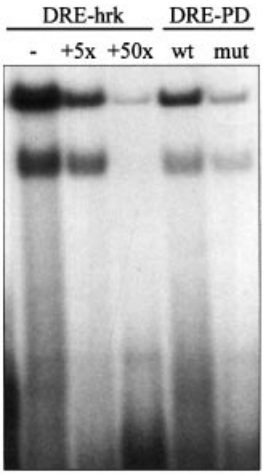

Fig. 1. The DRE sequence of the *hrk* gene binds to recombinant DREAM. Radiolabeled DRE-*hrk*, or wild-type (wt) and mutated (mut) DRE-PD probes were incubated with recombinant human DREAM, and the formation of the binding complex was analyzed by EMSA. For competition experiments, DREAM was pre-incubated with 5- and 50-fold molar excess of unlabeled DRE-*hrk* probe.

deprivation (Sanz *et al*., 2000). Since Hrk is sufficient to induce apoptosis in hematopoietic progenitor cells, we argued that its expression should be tightly controlled. Thus, we searched for regulatory elements in the mouse *hrk* gene and found a sequence in the 3′ UTR with significant homology to a gene silencer sequence named DRE (downstream regulatory element) located in the 5′ UTR of the prodynorphin gene (Carrion *et al*., 1998). As the DRE site binds to a repressor protein, DREAM (Carrion *et al*., 1999), we analyzed first the capacity of the sequence found in *hrk* to bind to recombinant human DREAM prepared from *Escherichia coli*. As shown in Figure 1, recombinant DREAM was able to bind to a radiolabeled DRE sequence from the *hrk* gene (DRE-*hrk*) as measured in electrophoretic mobility shift assays (EMSAs). Two specific DRE-*hrk*-retarded bands are competed by an excess (5- and 50-fold) of unlabeled probe. However, unlabeled irrelevant probes, including a random sequence of the same size and nucleotide composition, failed to compete off the DRE-*hrk* probe (data not shown). The presence of two bands is likely to be the result of either partial digestion or aggregated forms of the recombinant protein. As a control of binding specificity, recombinant DREAM was shown to bind to the wild-type (wt) DRE sequence of the prodynorphin gene (DRE-PD) but not to a mutated sequence (mut) (Figure 1).

Next, we analyzed the expression of DREAM in two IL-3-dependent hematopoietic progenitor cell lines, FL5.12 and FDCP-Mix, previously shown to upregulate Hrk after IL-3 deprivation (Sanz *et al*., 2000). Nuclear extracts from these cells were prepared and used in southwestern analysis with a radiolabeled DRE-*hrk* probe. As shown in Figure 2A, this probe reproducibly bound to two proteins of 34 and 110 kDa, which correspond to a monomer and a tetramer of DREAM, as previously characterized in another cell system (Carrion *et al*., 1999). These DNA–protein complexes were inhibited upon competition with an excess of unlabeled probe, but were not modified by an excess of unlabeled irrelevant

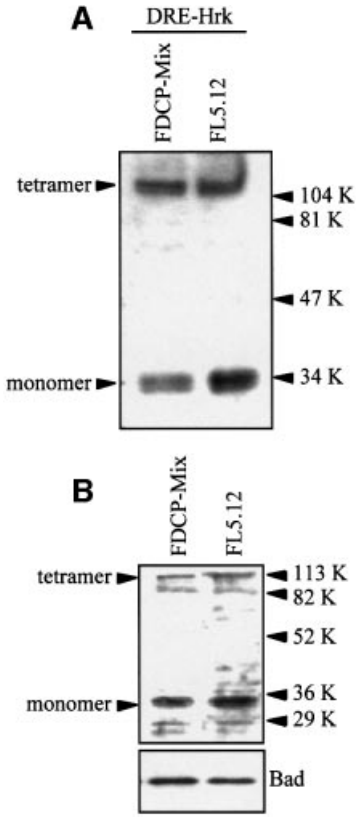

Fig. 2. DREAM is expressed in FDCP-Mix and FL5.12 hematopoietic progenitor cells. (**A**) Nuclear extracts were analyzed by southwestern blotting using a radiolabeled DRE-*hrk* probe. Arrowheads indicate the monomeric and tetrameric DREAM proteins. (**B**) The expression of DREAM protein was analyzed by western blotting with a specific goat antibody.

probe (data not shown). The expression of DREAM in FL5.12 and FDCP-Mix cells was confirmed further by western blotting with a specific antibody, and as shown in Figure 2B, both monomeric and tetrameric forms of DREAM were detected.

The DRE sequence of the hrk gene represses the expression of reporter genes

To examine the transcriptional-regulatory activity of the DRE-*hrk* sequence, we performed transient transfection assays with the heterologous promoter construct pCMV-EGFP containing either a DRE-*hrk* sequence or an irrelevant, nonsense sequence of the same size and nucleotide composition (DRE-NS) at the 3′ end of the EGFP cDNA. When we examined the FDCP-Mix cells 24 h after transfection with DRE-NS, 17.6% of the cells expressed EGFP, as determined by flow cytometry analysis (Figure 3). In contrast, cells transfected with the DRE-*hrk*-containing plasmid failed to express the EGFP protein (0.2%) showing the transcriptional repressor activity of this sequence. Since Hrk is expressed following IL-3 withdrawal, we tried to re-induce the expression of EGFP by incubating the transfected cells in the absence of IL-3. However, very little or no expression of EGFP protein was observed after growth factor deprivation (data not shown), most likely due to the inability of FDCP-Mix cells to activate the transcription of EGFP under a strong apoptotic stimulus. To confirm further the repressor activity of

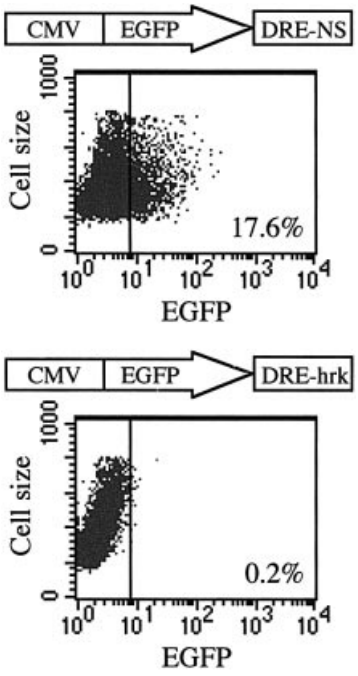

Fig. 3. The expression of EGFP is blocked when DRE-*hrk* is inserted downstream of the reporter cDNA. Flow cytometry analysis of FDCP-Mix cells after 24 h of transfection with the reporter plasmid pCMV-EGFP containing the DREAM binding sequence of the *hrk* gene (DRE-*hrk*) or a random, nonsense sequence of the same size and nucleotide composition (DRE-NS). DNA sequences were inserted downstream of the EGFP open reading frame. Dot plots are from a representative experiment (n = 3).

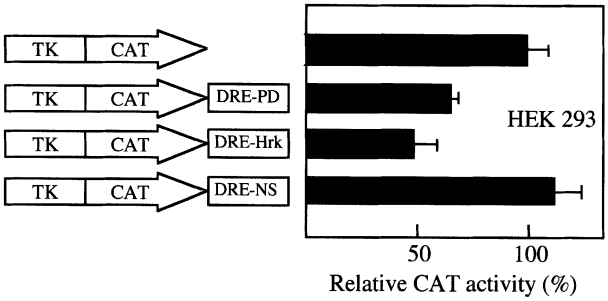

Fig. 4. The expression of CAT is inhibited when DRE-hrk is located at the 3′ end of the reporter cDNA. The reporter plasmid pTK-CAT containing the DREAM binding sequence of the *hrk* gene (DRE-*hrk*), or a random, nonsense sequence of the same size and nucleotide composition (DRE-NS), or the DRE sequence of the prodynorphin gene (DRE-PD), was introduced into HEK 293 cells. After 24 h of transfection, CAT activity was determined relative to that of the pTK-CAT reporter plasmid. Data (mean ± SD) were collected from three independent transfections.

DRE-*hrk*, HEK293 cells were transiently transfected with a different reporter construct, pTK-CAT, containing either a DRE-*hrk* sequence, a DRE sequence of the prodynorphin gene (DRE-PD) or an irrelevant sequence (DRE-NS) at the 3′ end of the CAT cDNA. Since HEK293 cells have very little or no endogenous DREAM (Carrion *et al*., 1999), the reporter plasmids were co-transfected with pCDNA3 containing the DREAM cDNA. As shown in Figure 4, the presence of DRE-*hrk* in the construct reduced the CAT activity by half [48.2 ± 9.7% (mean ± SD)] with respect to the pTK-CAT plasmid. In addition, CAT activity was also reduced when cells were transfected with the DRE-PD-containing plasmid, although to a lower extent (66.1 ± 3.6%), and no reduction in CAT activity was detected when DRE-NS was present in the construct. Therefore, these data show that DRE-*hrk* is able to reduce or abrogate the expression of heterologous genes when located at the 3′ end of the transcription unit.

Increase of cytoplasmic calcium and the absence of IL-3 inhibit binding of DREAM to the DRE-hrk sequence and induce Hrk expression

Since the transcriptional repression of DREAM has been shown to be regulated by calcium in human embryonic kidney 293 cells, we asked whether calcium mobilization could affect binding to DRE-*hrk* in the hematopoietic cell lines. FDCP-Mix and FL5.12 cells were cultured in the absence of IL-3, a well characterized stimulus for the induction of Hrk, or treated with the calcium ionophore ionomycin, and then nuclear extracts were prepared and

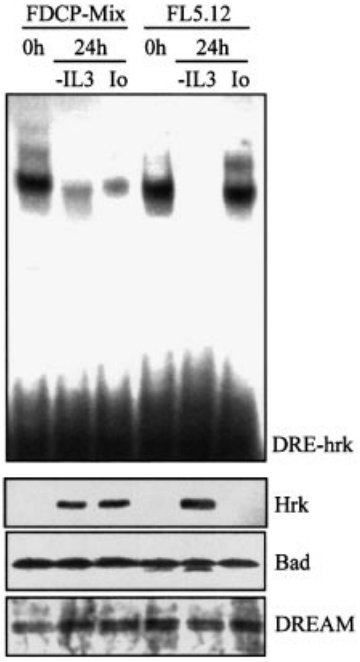

Fig. 5. Treatment with ionomycin blocks binding of DREAM to the DRE-*hrk* sequence and induces Hrk protein in FDCP-Mix myeloid progenitors. Cells were cultured in the absence of IL-3 or treated with ionomycin for 24 h, and then analyzed for the formation of the DREAM–DNA complex by EMSA using radiolabeled DRE-*hrk* (top), and for the expression of Hrk, Bad and DREAM proteins by western blotting (bottom).

analyzed in an EMSA (Figure 5). We showed first that the treatment of cells with IL-3 resulted in the formation of a DNA–protein complex when using a DRE-*hrk* probe, which was reduced after 6 h (not shown), and virtually lost after 24 h of IL-3 deprivation, indicating that DREAM was activated in response to IL-3. In addition, treatment of FDCP-Mix cells with ionomycin for 24 h significantly reduced the DREAM–DNA binding complex. Interestingly, ionomycin failed to affect the formation of the complex in FL5.12 cells in the same time period, although it was significantly decreased at longer incubation times (48 h) (not shown). In order to correlate the formation of

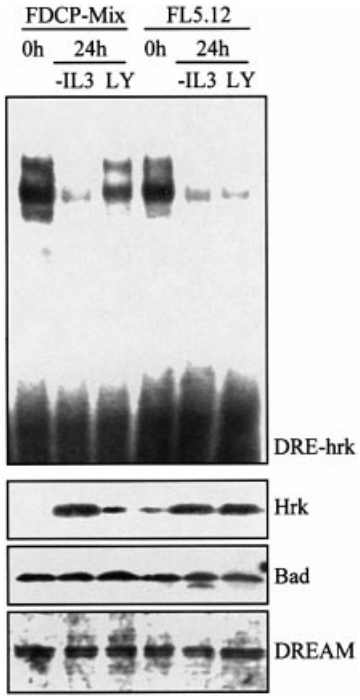

Fig. 6. Inactivation of PI 3-kinase inhibits binding of DREAM to DRE-*hrk* and induces Hrk protein in FL5.12 lymphoid progenitors. FDCP-Mix and FL5.12 cells were cultured in the absence of IL-3 or treated with LY294002 for 24 h. An EMSA was performed using a radiolabeled DRE-*hrk* probe to analyze the formation of the DREAM–DNA complex (top). The expression of Hrk, Bad and DREAM proteins was analyzed by western blotting (bottom).

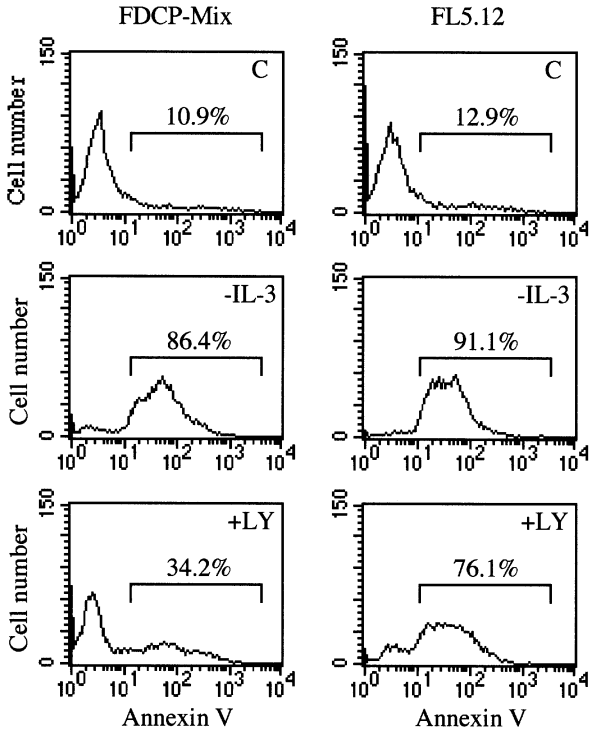

Fig. 7. Inactivation of PI 3-kinase induces apoptotic cell death. FDCP-Mix and FL5.12 cells were cultured in the absence of IL-3 or treated with LY294002 for 24 h, and then analyzed by flow cytometry with fluorescein isothiocyanate (FITC)-labeled annexin V. Numbers above the selected regions indicate the percentage of apoptotic cells.

DREAM–DNA binding complex with the expression of the *hrk* gene, we analyzed the levels of Hrk protein under the same culture conditions. As expected, the presence of IL-3 inhibited the expression of Hrk, which was upregulated after growth factor withdrawal (Figure 5). Consistent with the different DREAM–DNA binding pattern observed in the two cell lines, treatment with ionomycin for 24 h induced the expression of Hrk in FDCP-Mix but not FL5.12 cells, although an incubation time of 48 h significantly upregulated Hrk in FL5.12 (not shown). Consistent with previously described data (Sanz *et al*., 2000), expression of Hrk correlated with induction of apoptosis in both cell lines (data not shown). Interestingly, none of the treatments modified significantly the protein (Figure 5) and mRNA (data not shown) levels of DREAM, suggesting that the activity of DREAM is not transcriptionally regulated.

DREAM is activated through a PI 3-kinase-dependent pathway

PI 3-kinase is recruited and activated during the intracellular signal transduction of many receptors and has been implicated in the signaling of survival factors such as IL-3 (Yao and Cooper, 1995; Franke *et al*., 1997). Since calcium mobilization does not seem to regulate efficiently the *hrk*-binding capacity of DREAM in FL5.12 cells, we studied whether a PI 3-kinase-dependent pathway could contribute to the formation of the binding complex. FDCP-Mix and FL5.12 cells were incubated for 24 h with LY294002, a specific inhibitor of PI 3-kinase, and the DNA binding activity of DREAM was assessed by EMSA. As shown in Figure 6, LY294002 reduced binding to DRE-*hrk* in both cell lines. Interestingly, while the binding complex was only slightly reduced in FDCP-Mix, it almost disappeared in FL5.12 cells. This pattern of DREAM–DNA complex formation was consistent with the expression of Hrk protein, which was slightly induced in FDCP-Mix and clearly upregulated in FL5.12 cells, further indicating the Hrk repressor activity of DREAM. As previously shown in cells treated with ionomycin, the expression of DREAM remained constant following treatment with LY294002 (Figure 6). Moreover, a clear correlation was observed between the upregulated expression of Hrk and the increase in the number of apoptotic cells as determined by flow cytometry analysis with annexin V (Figure 7). By 24 h of treatment with LY294002, 34.2% of FDCP-Mix cells were annexin V positive, whereas most of the FL5.12 cells (76.1%) were apoptotic within the same time period. Similar results were obtained when cells were treated with wortmannin, another specific inhibitor of PI 3-kinase (data not shown). As expected, in the absence of IL-3 most of the cells (~90%) were apoptotic (Figure 7).

DREAM is phosphorylated by a PI 3-kinase-dependent and Akt-independent pathway

These results prompted us to analyze whether DREAM was phosphorylated in response to IL-3. In these experiments, FL5.12 cells were stimulated with IL-3 in the presence or absence of LY294002, and the phosphorylation of endogenous DREAM was assayed in DREAM

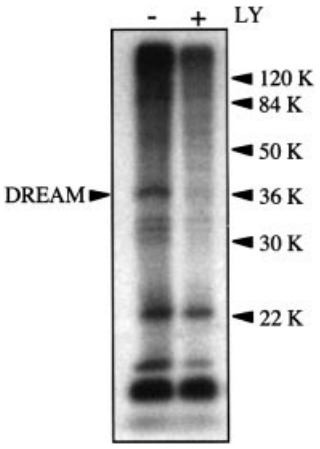

Fig. 8. DREAM is phosphorylated by a PI 3-kinase-dependent pathway. FL5.12 cells were cultured in the presence of [^{32}P]ortho-phosphate with or without LY294002. After labeling, cells were lysed and DREAM was immunoprecipitated using a specific polyclonal antibody and protein A/G–agarose beads. Phosphorylation of DREAM was determined by autoradiography.

immunoprecipitates. As shown in Figure 8, stimulation with IL-3 induced phosphorylation of DREAM, which was abrogated when cells were treated with the PI 3-kinase inhibitor (Figure 8). Thus, IL-3 induced DREAM phosphorylation through a PI 3-kinase-dependent pathway in hematopoietic progenitor cell lines, which may allow DREAM to bind to the *hrk* gene.

Because Akt plays an important role in anti-apoptotic PI 3-kinase signaling (del Peso *et al*., 1997), we studied whether this serine/threonine kinase was able to phosphorylate DREAM. However, we could not find a consensus phosphorylation site for Akt within the DREAM amino acid sequence, making this protein an unlikely candidate for Akt phosphorylation. To determine experimentally whether Akt could phosphorylate DREAM, recombinant DREAM was incubated with Akt immunoprecipitates from FDCP-Mix and FL5.12 cells, and consistent with the previous finding, Akt failed to phosphorylate DREAM (data not shown).

Discussion

Since the expression of Hrk is sufficient to induce apoptosis in hematopoietic progenitors, it is likely that this gene is tightly controlled at the transcriptional level to avoid inappropriate cell death within the hematopoietic compartment. Here we have shown the presence of a DREAM binding sequence in the 3′ UTR of *hrk* mRNA, DRE-*hrk*. This sequence specifically binds to the transcriptional repressor DREAM in cells cultured with IL-3, and this binding is lost in the absence of growth factor, suggesting the existence of a DREAM regulatory pathway triggered by IL-3. A DREAM binding sequence has been described recently as a gene silencer located in the 5′ UTR of the human prodynorphin gene (Carrion *et al*., 1998). Interestingly, our results show that the DREAM binding sequence found in *hrk* inhibits transcription when located downstream of the open reading frame. DNA sequences that act as silencers when located in the 3′ region of the transcription unit have been described for a number of genes, including the serine protease inhibitor 2.3 and neuronal genes (Paul *et al*., 1998; Thiel *et al*., 1998), but not for genes involved in the execution of apoptosis. Thus, the mechanism of transcriptional inhibition described here represents a novel regulatory mechanism for pro-apoptotic members of the Bcl-2 family that is triggered by IL-3 in hematopoietic progenitors. It is interesting that IL-3 also induces the expression of the anti-apoptotic protein Bcl-x_L through activation of transcriptional factors such as Stat5 and Stat3 (Catlett-Falcone *et al*., 1999; Kieslinger *et al*., 2000), which indicates that IL-3 activates a transcriptional pathway that leads to the expression of an anti-apoptotic protein, and also a repressor pathway that blocks the expression of a highly lethal protein. It has been described previously that Bax, an apoptosis-promoting member of the Bcl-2 gene family, can be transcriptionally repressed by the Gfi-1 proto-oncogene through direct binding to the *bax* promoter in IL-2-dependent T-cell lines (Grimes *et al*., 1996). However, in contrast to Hrk, Bax is normally expressed in most hematopoietic cells, including T cells (Grimes *et al*., 1996; Sanz *et al*., 2000). Thus, although transcriptional repression of Bax may contribute to balance the ratio between pro-apoptotic and anti-apoptotic members of the Bcl-2 family, the expression of Bax is not sufficient to induce apoptosis. To this end, it has been shown that hematopoietic and non-hematopoietic cells can be stably transfected with Bax (Kobayashi *et al*., 1998; Sawada *et al*., 2000). In contrast, we have been unable to obtain cells stably overexpressing Hrk (Sanz *et al*., 2000; our unpublished data).

We have also shown that the *hrk* transcriptional repressor can be regulated by different signaling pathways, including phosphorylation and calcium mobilization. Binding of DREAM to the DRE-*hrk* sequence can be inhibited in FDCP-Mix cells following treatment with ionomycin, whereas the binding complex is impaired in FL5.12 cells treated with PI 3-kinase inhibitors. It has been shown that when intracellular calcium concentration rises, DREAM binds to calcium and releases the DRE sequence, permitting a higher level of promoter activity (Carrion *et al*., 1999). Thus, an increase in cytosolic calcium, which has been implicated in apoptosis (McConkey *et al*., 1989; Caron-Leslie *et al*., 1991), may enable the expression of Hrk through inactivation of its transcriptional repressor. In addition, phosphorylation might play an important role in the activation of DREAM in hematopoietic progenitors. Unlike other transcriptional repressors (ERF, Bcl-6 and HES-1) that have been shown to be inactivated by phosphorylation (Strom *et al*., 1997; Niu *et al*., 1998; Le Gallic *et al*., 1999), DREAM seems to exert its repressor activity when it is phosphorylated by a PI 3-kinase-dependent pathway. Thus, mitogenic signals may inactivate transcriptional repressors (ERF, HES-1) that control genes involved in cell growth, whereas the same or other mitogenic signals may activate transcriptional repressors (DREAM) that block the expression of cell death genes. It is not clear why DREAM is preferentially regulated by calcium in FDCP-Mix myeloid progenitors and by phosphorylation in FL5.12 pre-B cells. A likely explanation is that different apoptotic members of the Bcl-2 family, mainly the BH3-only proteins, are required to execute particular death responses in individual cell types. Consistent with this is the observation that pre-B cells, as well as pre-T cells, from

mice deficient in the BH3-only protein Bim are refractory to apoptosis induced by calcium flux, indicating that Bim must be the dominant transducer of this cytotoxic signal in lymphocytes (Bouillet *et al*., 1999). In this regard, it would be interesting to analyze the expression of Hrk in Bim-deficient lymphoid cells treated with calcium mobilization agents and other apoptotic stimuli in order to shed light on the participation of Hrk in the survival/apoptosis of this cell lineage.

In conclusion, several BH3-only proteins are expressed in viable cells but are maintained in a latent state until activated by apoptotic signals (Li *et al*., 1998; Puthalakath *et al*., 1999). By contrast, Hrk is not expressed in viable cells and is induced only after an apoptotic stimulus. We describe here a transcriptional repression mechanism triggered by IL-3 that at least may contribute to block the expression of Hrk and avoid apoptosis in hematopoietic progenitor cell lines. Further studies will need to address the physiological relevance of Hrk transcriptional regulation within the hematopoietic system. It will be of special interest to find other target genes of DREAM that may be involved in proliferation and survival.

Materials and methods

Cell culture

FL5.12 and FDCP-Mix cell lines were grown in RPMI 1640 medium (Seromed Biochrom KG, Berlin, Germany), supplemented with 10% fetal calf serum (FCS) and 10% Wehi3B culture supernatant as an IL-3 source. Human embryonic kidney 293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS. When indicated, cell lines were treated with 20 μM LY294002 (Sigma, St Louis, MO), a specific inhibitor of PI 3-kinase, or 1 μM ionomycin (SIGMA), a carrier of calcium across membranes. The apoptotic cells were detected with annexin V labeled with FITC (Pharmingen, San Diego, CA) by flow cytometry.

Gene reporter assays

A double-stranded oligonucleotide from the 3′ UTR of the *hrk* gene (DRE-*hrk*; 5′-TCGAGTTAACGAAACACAGACAGAGGAAGCCCC-TCGGGAG-3′) or a nonsense double-stranded sequence of the same size and nucleotide composition (DRE-NS; 5′-TCGAGTTAACGG-ACCAAAAAGCAGCAGAGACACTCGCGGG-3′) containing a *Xho*I site at 5′ was cloned into the *Xho*I and *Bst*1107I sites of pCMV-EGFP vector (Clontech, Palo Alto, CA). Cloning was confirmed by linearization of the construct with *Hpa*I, incorporated as a unique site at the 5′ end of the insert, and dideoxy sequencing. FDCP-Mix cells (5×10^6) were transfected by electroporation as previously described (Sanz *et al*., 2000). After 24 h of transfection, cells were analyzed for expression of green fluorescent protein by flow cytometry using a FACScan analyzer (Becton Dickinson, San Jose, CA). The reporter plasmids pTK-CAT containing the sequences described above or the DREAM binding sequence of the prodynorphin gene at the 3′ end of the CAT cDNA were transfected into 293 cells by calcium phosphate precipitation as previously described (Carrion *et al*., 1999). Briefly, a mixture of DNA containing 3 μg of reporter plasmid and DREAM cDNA cloned in pCDNA3, 1 μg of β-galactosidase expression vector (Pharmacia, Uppsala, Sweden) and 6 μg of carrier plasmid DNA was coprecipitated with calcium phosphate and added to the 293 cells. After 12 h, cells were washed and left for an additional period of 24 h with fresh medium. The chloramphenicol acetyltransferase (CAT) activity was assayed in 100 μg of protein extract and normalized with respect to β-galactosidase activity.

Protein analysis

The expression of Hrk was determined by western blotting as previously described (Silva *et al*., 1996). Blots were incubated with rabbit antibodies against Hrk (Sanz *et al*., 2000) and Bad (Santa Cruz Biotechnology, Santa Cruz, CA), or goat antibodies against DREAM (Santa Cruz Biotechnology), and then incubated with secondary antibodies conjugated to alkaline phosphatase (Tropix, Bedford, MA). Bound antibodies were detected by a chemiluminescence system (Tropix).

Southwestern analysis was carried out as described previously (Carrion *et al*., 1998). Briefly, nuclear proteins (50 μg) were resolved in SDS–10% polyacrylamide gels and transferred to nitrocellulose membranes. The blots were renatured in phosphate-buffered saline, blocked with 3% non-fat dry milk, and then incubated for 2 h in the presence of ^{32}P-labeled double-stranded DRE-*hrk* oligonucleotide (10^6 c.p.m./ml). After washing to remove the unbound probe, blots were exposed for autoradiography.

In vivo phosphorylation assay

FDCP-Mix cells were starved of FCS for 12 h, cultured for 1 h in phosphate-free RPMI containing 20 μM LY294002, and then incubated in the presence of [^{32}P]orthophosphate (100 μCi/ml) for 2 h. Cells were lysed and endogenous DREAM was immunoprecipitated with peptide-specific DREAM polyclonal antibody (J.R.Naranjo, B.Mellstrom and W.A.Link, in preparation). Immunocomplexes were collected with protein A–Sepharose, resolved by 9% SDS–PAGE and analyzed by autoradiography.

Electrophoretic mobility shift assays

Cells were cultured for different periods of time in the presence or absence of ionomycin or LY294002, and were then lysed as previously described (Silva *et al*., 1999). In brief, nuclear extracts (5 μg of total protein) were incubated with ^{32}P-labeled double-stranded DRE-*hrk* oligonucleotide, and then run on a 5% non-denaturing polyacrylamide gel. Gels were dried and visualized by autoradiography. In some experiments, 50 ng of recombinant DREAM were incubated with the labeled DRE-*hrk* probe or a labeled probe from the promoter of the prodynorphin gene (DRE-PD), containing a wild-type or a mutated DRE sequence (Carrion *et al*., 1999). For competition assays, the recombinant protein was pre-incubated with 5- and 50-fold molar excess of unlabeled DRE-*hrk* probe.

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