

Fas signaling and blockade of Bcr-Abl kinase induce apoptotic Hrk protein via DREAM inhibition in human leukemia cells

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Background and Objectives. The apoptotic Bcl-2 family member Hrk is transcriptionally silenced via DREAM in hematopoietic progenitor cell lines, and is specifically induced after growth factor withdrawal. Given that expression of Hrk is sufficient to induce apoptosis, we studied the expression of this apoptotic protein and its regulatory mechanism in human leukemia cells.

Design and Methods. K562 chronic myeloid leukemia cells were treated with STI571, a Bcr-Abl kinase inhibitor, and the Jurkat T-cell leukemia cell line was incubated with agonistic anti-Fas antibodies. Following treatment, we correlated the expression of Hrk protein with the DNA binding capacity of DREAM, and the induction of apoptosis.

Results. We show that treatment of K562 with STI571 blocks the binding of DREAM to the Hrk gene and allows the expression of Hrk, which correlates with the induction of apoptosis. Similarly, treatment of Jurkat cells with agonistic anti-Fas antibodies triggers the expression of Hrk through DREAM inactivation. Interestingly, inhibition of caspases, by culturing Jurkat cells in the presence of z-VAD-fmk, abrogates Fas-mediated Hrk expression and apoptosis. Furthermore, *in vitro* analysis shows that active recombinant caspase-3 releases a fragment from the DREAM protein, suggesting that caspase-3 may be upstream of DREAM.

Interpretation and Conclusions. These data suggest that apoptosis inducers as diverse as oncoprotein inhibitors and cell death receptor activators trigger Hrk expression via blockade of DREAM in leukemia cells, and this apoptotic pathway may be regulated, at least in some systems, by the proteolytic activity of caspase-3.

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In mammals the executionary arm of apoptosis involves a family of death proteases, called caspases, that are activated in a proteolytic cascade to execute the cell death program.¹ The activation of upstream caspases represents a critical checkpoint in the decision of whether a cell will survive or die.² Upstream caspases are controlled by several molecules, including proteins of the Bcl-2 family. Some pro-apoptotic members of this family, such as Bik/Nbk, Blk, Bad, Bid, Bim and Hrk, contain only the BH3 *cell death* motif (BH3-only proteins) that acts as a dimerization domain for anti-apoptotic proteins (i.e., Bcl-2 and Bcl-x_L).³ The pro-apoptotic activity of these BH3-only proteins is mediated by the BH3 region, as deletion or mutation of this region abrogates their binding to Bcl-2 and Bcl-x_L and their killing activity.⁴ Furthermore, the BH3-only proteins have been suggested to represent the physiologic antagonists of anti-apoptotic Bcl-2 family members.⁵ We have previously shown that Hrk is specifically induced in murine myeloid and lymphoid progenitor cell lines after growth factor withdrawal, and that this induction is sufficient to trigger apoptosis, as the ectopic expression of Hrk provokes cell death in hematopoietic progenitors even in the presence of survival factors (i.e., interleukin-3).⁶ More recently, we identified a silencer sequence in the 3' untranslated region of the Hrk gene (DRE-hrk) that binds to the transcriptional repressor DREAM⁷ in interleukin-3-dependent murine hematopoietic cell lines, which contributes to avoiding inappropriate expression of Hrk and consequently apoptosis in these cells.⁸ As treatment with chemotherapeutic drugs and signaling through the cell death receptor Fas trigger apoptosis in leukemia cells,^{9,10} we studied whether Hrk and its regulatory mechanism play any role in these apoptotic pathways. We treated human leukemia cells with STI571, a Bcr-Abl kinase inhibitor, or agonistic Fas antibodies, and found that both apoptotic stimuli induced Hrk protein and blocked the DNA binding activity of DREAM. Furthermore, we showed in Jurkat cells that caspase-3 may be upstream of

DREAM and therefore, may be involved in the expression of Hrk.

Design and Methods

Cell culture

Jurkat and K562 cell lines were grown in RPMI 1640 medium (Seromed Biochrom KG, Berlin, Germany) and supplemented with 10% fetal calf serum. When indicated, cell lines were treated with 100 ng/mL anti-Fas antibody (CH11) (Upstate, Charlottesville, VA, USA), 2 μ M STI571, a Bcr-Abl kinase inhibitor, or anti-Fas plus 100 μ M z-VAD-fmk (Roche, Basel, Switzerland), a broad spectrum caspase inhibitor. Viability and total cell counts were determined by trypan blue exclusion and counting of at least 200 cells from each individual culture.

Protein analysis

The expression of Hrk was determined by Western blotting as previously described.⁶ Blots were incubated with rabbit antibodies against Hrk,⁶ and the p85 proteolytic fragment of poly ADP-ribose polymerase (PARP) (Promega, Madison, WI, USA), mouse antibodies to Cpp32 (procaspase-3) (BD Biosciences, Heidelberg, Germany), and β -tubulin (SIGMA, St. Louis, MO, USA), and goat anti-DREAM antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and then incubated with secondary antibodies conjugated to alkaline phosphatase (Tropix, Bedford, MA, USA). Bound antibodies were detected by a chemiluminescence system (Tropix).

In vitro digestion of DREAM

Recombinant DREAM⁸ (2 μ g) was incubated with 60 ng of active recombinant caspase-3 (Calbiochem, Darmstadt, Germany) in assay buffer (100 mM NaCl, 10 mM DTT, 1 mM EDTA, 10% glycerol, 0.1% Chaps, and 50 mM HEPES pH 7.4) for 3 h at 30°C, and then analyzed by Western blotting with antibodies against DREAM.

Electrophoretic mobility shift assays (EMSA)

Nuclear extracts (5 μ g of total protein) were incubated with ³²P-labeled double-stranded DRE-hrk oligonucleotide, and then run on a 5% non-denaturing polyacrylamide gel as previously described.⁸ For competition assays, nuclear extracts were pre-incubated with a 100-fold molar excess of unlabeled DRE-hrk or an irrelevant probe. Gels were dried and visualized by autoradiography.

Results and Discussion

We have previously shown that Hrk is not expressed in viable hematopoietic progenitors, but

that it is specifically induced at the mRNA and protein level after growth factor deprivation.⁶ Lack of Hrk expression is mediated, at least in part, by a mechanism that involves the transcriptional repressor DREAM.⁸ Since Hrk is sufficient to trigger apoptosis in hematopoietic progenitors, we asked whether the expression of this apoptotic protein was induced in human leukemia cells by inhibition of Bcr-Abl kinase activity and treatment with agonistic Fas antibodies. First, we analyzed the expression of Hrk and the DNA binding activity of DREAM in K562 cells treated with STI571, a Bcr-Abl kinase inhibitor that has been shown to eradicate human chronic myeloid leukemia cells efficiently.¹¹ Nuclear extracts were prepared and used to analyze the binding of DREAM to a radiolabeled Hrk probe that contains the DREAM binding site (DRE-hrk). A pattern of DNA-protein binding similar to that described in other systems⁸ was detected in untreated K562 cells, whereas it was reduced after 6 h (not shown), and virtually lost after 24 h of STI571 treatment (Figure 1A). To ensure the specificity of the binding complex, we demonstrated that the DRE-hrk retarded band was competed for by an excess (100-fold) of unlabeled probe; however, an unlabeled irrelevant probe failed to compete with the DRE-hrk probe (Figure 1A). These data indicate that DREAM is inactivated in response to STI571. In order to correlate the formation of a 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, untreated cells did not express Hrk, but following treatment with STI571, Hrk was clearly induced (Figure 1B). Consistent with previously described data,⁶ expression of Hrk correlated with induction of apoptosis in STI571-treated K562 as assessed by detection of the p85 fragment of PARP that results from caspase-3 cleavage of the intact protein (Figure 1B). It has been shown that treatment of K562 cells with STI571 suppresses the Stat5-dependent expression of the anti-apoptotic protein Bcl-x_L.¹² Our results extend these data and suggest that both loss of Bcl-x_L and induction of Hrk may be orchestrated by the same apoptotic pathway, which disrupts the equilibrium between anti-apoptotic and pro-apoptotic proteins within the STI571-treated leukemic cells.

Engagement of the plasma membrane receptor Fas can induce apoptosis of leukemic cells,¹³ and mediates the ability of cytotoxic T-lymphocytes and natural killer cells to eliminate tumor cells.¹⁴ However, it is not known whether endogenous expression of Hrk is regulated through Fas signal-

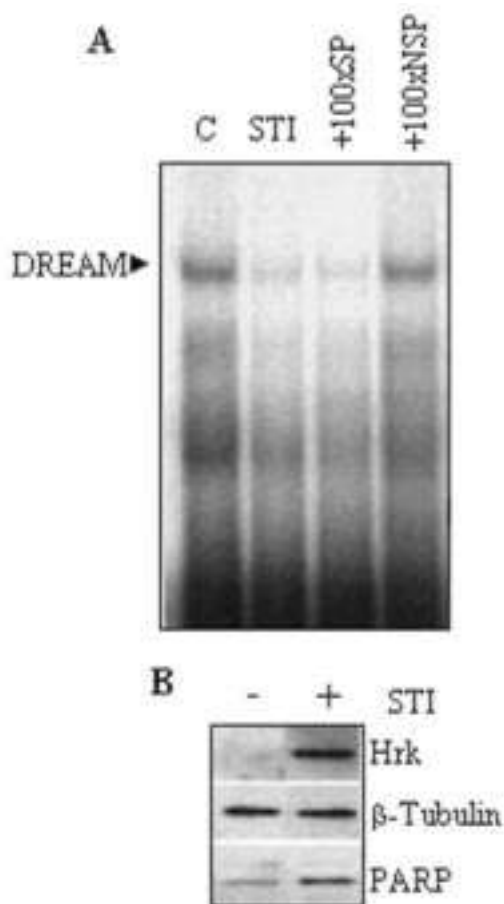


Figure 1. Expression of Hrk protein and DNA-binding activity of DREAM in K562 cells treated with STI571. (A) Cells were cultured with (+) or without (-) STI571 for 24 h, and then analyzed for the formation of the DREAM-DNA complex by EMSA using radiolabeled DRE-hrk. For competition experiments, DREAM was pre-incubated with 100-fold molar excess of unlabeled DRE-hrk probe (100xSP) or an irrelevant non-specific probe (100xNSP). (B) The expression of Hrk and the generation of the p85 fragment of PARP were analyzed by Western blotting. The levels of β -tubulin were also analyzed to assure equal loading.

ing. Here, we show that Hrk protein is induced in Jurkat T-cell leukemia cells treated with an agonistic Fas antibody (Figure 2), which correlates with a decreased cell viability (29% of viable cells) and activation of caspase-3, a downstream caspase of the apoptosis pathway, as assessed by Western blot analysis with an antibody that recognizes the proenzyme but not the processed active caspase-3 (Figure 2). Consistent with our previous data (Figure 1A)⁸ expression of Hrk correlated with loss of

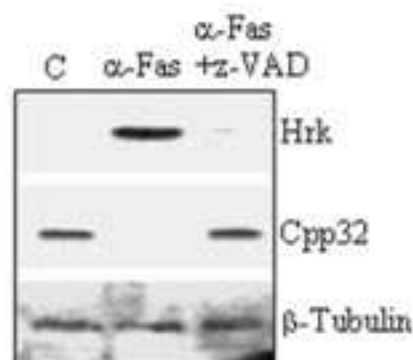


Figure 2. Expression of Hrk in Jurkat cells treated with an agonistic Fas antibody. Cells were treated with a Fas-activating antibody for 24 hours in the absence or in the presence of z-VAD-fmk. The expression of Hrk and the generation of active caspase-3 (not recognized by anti-Cpp32) was determined by Western blotting using specific antibodies for Hrk. The levels of β -tubulin were also analyzed to assure equal loading.

DREAM-DNA binding complex, which was demonstrated to be specific by competition assays with unlabeled DRE-hrk or an irrelevant probe (Figure 3). These data indicate that Fas and inhibition of Bcr-Abl kinase may induce Hrk through the same transcriptional mechanism.

It has been shown that overexpression of Bcl-2 and Bcl-x_L represses the induction of Hrk in hematopoietic progenitors,⁶ and blocks Fas-mediated apoptosis in type II cells (ie., CEM, Jurkat).¹⁵ As both anti-apoptotic proteins control the activation of caspases, we asked whether inhibition of these proteases would have any effect on the expression of Hrk. Figure 2 shows that in the presence of z-VAD-fmk, a cell-permeable broad spectrum inhibitor of caspases, Fas signaling was unable to induce expression of Hrk and to generate active caspase-3 in Jurkat cells. Interestingly, z-VAD-fmk hampered the Fas-mediated inhibition of the DREAM-DNA complex (Figure 3), which suggests that caspase-3 is upstream of DREAM, and activation of the protease may release DREAM from the silencer sequence in the Hrk gene, allowing the expression of Hrk. Consistently, it has been recently described that DREAM is a substrate for caspase-3.¹⁶ To confirm this model further, recombinant DREAM was incubated with active recombinant caspase-3 and then analyzed by Western blotting with an antibody against DREAM. As shown in Figure 4, this treatment resulted in the generation of a cleavage fragment with a molecular size (25-28 kDa) similar to that previously

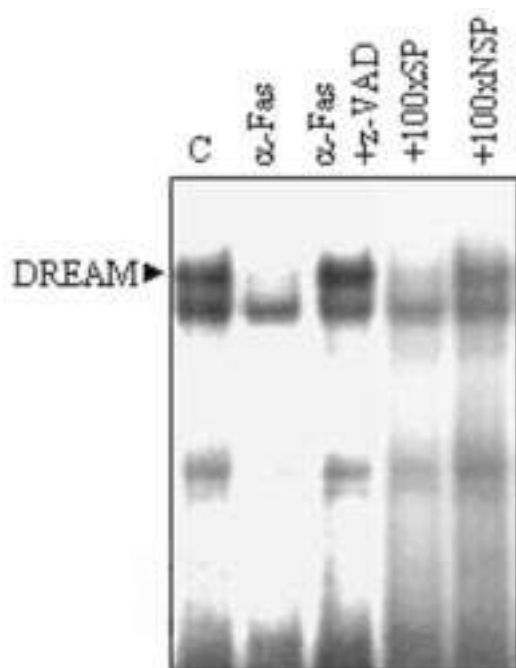


Figure 3. DNA-binding activity of DREAM in Jurkat cells treated with an agonistic Fas antibody. Nuclear extracts from untreated cells or cells treated for 24 h with anti-Fas in the presence or in the absence of z-VAD-fmk were analyzed for the formation of the DREAM-DNA complex by an EMSA using radiolabeled DRE-hrk. For competition experiments, DREAM was pre-incubated with a 100-fold molar excess of unlabeled DRE-hrk probe (100xSP) or an irrelevant non-specific probe (100xNSP).

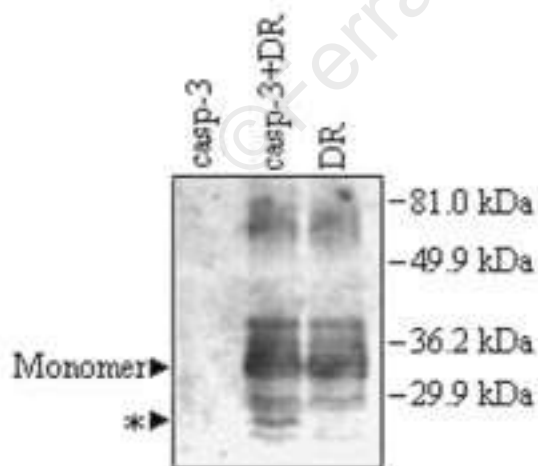


Figure 4. *In vitro* cleavage of DREAM by caspase-3. Recombinant DREAM (DR) was incubated with active recombinant caspase-3 (casp-3) for 3 h and then analyzed by Western blotting using an anti-DREAM antibody. High molecular weight bands correspond to aggregated forms of DREAM. Arrowheads indicate the monomer of DREAM and a proteolytic fragment (*).

described.¹⁶ This is an intriguing result as data so far available show that BH3-only proteins cause cytochrome c release and caspase activation in response to apoptotic stimuli.¹⁷ A likely explanation is that Hrk is induced in Jurkat cells undergoing apoptosis as a way to amplify or to assure the progression of the apoptotic process. Consistent with this hypothesis, upon activation of Fas, the BH3-only protein Bid is cleaved by caspase-8, an upstream caspase, and the truncated Bid translocates to mitochondria and induces cytochrome c release and activation of caspase-3.^{18,19} Therefore, in this model initiation of apoptosis would be Hrk-independent but following activation of caspase-3 via Bid, Hrk would be transcriptionally induced. This caspase-3-dependent expression of Hrk was not detected in STI571-treated K562 cells.

In conclusion, we show that apoptotic stimuli as diverse as inhibition of the Bcr-Abl kinase, and cell death receptor engagement trigger a signaling pathway in leukemic cells that leads to the expression of Hrk through inactivation of DREAM, which may be mediated, at least in Jurkat cells, by the proteolytic activity of caspase-3.

Contributions and Acknowledgments

All the authors contributed to the conception of the experiments to be done, the experimental work, and the revision of the manuscript and all approved the final version. We thank Novartis Inc., Basel, Switzerland, for providing the STI571.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

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PEER REVIEW OUTCOMES

Manuscript processing

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What is already known on this topic

Proteins of the Bcl2 family including Hrk are able to induce cell death when ectopically expressed. Binding of Hrk to the transcriptional repressor DREAM avoids inappropriate Hrk expression and apoptosis.

What this study adds

Treatment of K562 and Jurkatt cell lines with STI571 or agonistic Fas antibodies induced Hrk expression and blocked DNA binding activity of DREAM. This provides important insights into apoptotic pathways and triggering by biological agents.

Potential implications for clinical practice

Increasing understanding of apoptotic pathways and mediators might be relevant to the design of new agents in modern antileukemic therapy.

Francesco Lo Coco, Associate Editor