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Physical and functional interaction between BH3-only protein Hrk and mitochondrial pore-forming protein p32

J Sunayama[1,2,3], Y Ando[2], N Itoh[2], A Tomiyama[2], K Sakurada[2], A Sugiyama[3], D Kang[4], F Tashiro[3], Y Gotoh[1], Y Kuchino[2,5,6] and C Kitanaka*[,2,7]

[1] Institute of Molecular and Cellular Biosciences, University of Tokyo, Tokyo, Japan;
[2] Biophysics Division, National Cancer Center Research Institute, Tokyo, Japan;
[3] Department of Biological Science and Technology, Faculty of Industrial Science and Technology, Tokyo University of Science, Chiba, Japan;
[4] Department of Clinical Chemistry and Laboratory Medicine, Kyushu University Graduate School of Medical Sciences, Fukuoka, Japan;
[5] Laboratory of Pharmacognosy and Phytochemistry, School of Pharmaceutical Sciences, Showa University, Tokyo, Japan;
[6] CREST, Japan Science and Technology Corporation, Saitama, Japan;
[7] Biology Division, National Cancer Center Research Institute, Tokyo, Japan
* Corresponding author: C Kitanaka, Biology Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chu-o ku, Tokyo 104-0045, Japan. Tel: +81-3-3542-2511; Fax: +81-3-3542-0807; E-mail: ckitanak@ncc.go.jp

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Abstract

Bcl-2 homology domain (BH) 3-only proteins of the proapoptotic Bcl-2 subfamily play a key role as initiators of mitochondria-dependent apoptosis. To date, at least 10 mammalian BH3-only proteins have been identified, and it is now being realized that they have different roles and mechanisms of regulation in the transduction of apoptotic signals to mitochondria. Hrk/DP5 is one of the mammalian BH3-only proteins implicated in a variety of physiological and pathological apoptosis, yet the molecular mechanism involved in Hrk-mediated apoptosis remains poorly understood. In an attempt to identify cellular proteins participating in Hrk-mediated apoptosis, we have conducted yeast two-hybrid screening for Hrk-interacting proteins and isolated p32, a mitochondrial protein that has been shown to form a channel consisting of its homotrimer. *In vitro* binding, co-immunoprecipitation, as well as immunocytochemical analyses verified specific interaction and colocalization of Hrk and p32, both of which depended on the presence of the highly conserved C-terminal region of p32. Importantly, Hrk-induced apoptosis was suppressed by the expression of p32 mutants lacking the N-terminal mitochondrial signal sequence (p32(74–282)) and the conserved C-terminal region (p32 (1–221)), which are expected to inhibit binding of Hrk competitively to the endogenous p32 protein and to disrupt the channel function of p32, respectively. Furthermore, small interfering RNA-mediated knockdown of p32 conferred protection against Hrk-induced apoptosis. Altogether, these results suggest that p32 may be a key molecule that links Hrk to mitochondria and is critically involved in the regulation of Hrk-mediated apoptosis.

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Abbreviations: BH, Bcl-2 homology domain; NGF, nerve growth factor; JNK, c-jun N-terminal kinase; GAL4AD, GAL4 activation domain; GAL4DBD, GAL4 DNA-binding domain; GST, glutathione *S*-transferase; TM, transmembrane; siRNA, small interfering RNA; PT, permeability transition; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; VDAC, voltage-dependent anion channel; PBS, phosphate-buffered saline; BSA, bovine serum albumin; GFP, green fluorescent protein

Introduction

The Bcl-2 protein family, composed of members that promote (proapoptotic) and inhibit (antiapoptotic) apoptosis, plays a central role in apoptosis regulation through the modulation of the permeability of the mitochondrial membrane against apoptogenic molecules such as cytochrome *c* and Smac/DIABLO.[1–4] The proapoptotic members of the Bcl-2 family are further categorized into two subgroups, those having Bcl-2 homology (BH) domains 1–3 ('multidomain' members, represented by Bax and Bak) and those having only the BH3 domain ('BH3-only' members, represented by Bid, Bad, Bik, and Hrk).[4] Accumulating evidence suggests that the BH3-only members and the multidomain members have distinct roles in apoptosis regulation, acting, respectively, as initiators and downstream signal transducers of apoptosis: upon receiving apoptotic stimuli, the BH3-only members are activated primarily via transcriptional and post-translational mechanisms, which then activate the downstream multidomain members to permeabilize the mitochondrial membrane.[4–7] In contrast to the case of *Caenorrhabditis elegans*, whereby the only BH3 protein EGL-1 serves as the initiator for all developmental apoptosis of somatic cells, mammals have at least 10 BH3-only proteins that differ in their expression pattern and mode of activation, which may imply that, in mammals, each BH3-only protein functions as a stimuli- or cell type-specific initiator of apoptosis.[8]

Hrk/DP5 is one of the mammalian BH3-only proteins identified during screening for proteins that interact with Bcl-2 or are upregulated in nerve growth factor (NGF) deprivation-induced death of sympathetic neuron.[9,10] The initial studies demonstrated that Hrk expression is relatively restricted to the brain and in the lymphoid tissues,[9,10] suggesting that Hrk may have a specific role in these tissues. Consistent with this idea,

induction of Hrk expression has been demonstrated in NGF-deprived sympathetic neurons,[9] in apoptosis of cortical neurons after amyloid-β protein exposure,[11] in apoptotic cerebellar granule neurons cultured under a low potassium condition,[12] in spinal neurons of amyotrophic lateral sclerosis patients,[13] in retinal ganglion cells of axontomized retina,[14] as well as in growth factor-deprived hematopoietic cells.[15] Although Hrk expression has also been detected in blastomere undergoing fragmentation during embryonic development,[16] these characteristic patterns of Hrk expression strongly suggest that this BH3-only protein may have a specific role in initiating neuronal and hematopoietic cell apoptosis under physiological and pathological conditions.

Despite such potential significance of Hrk in physiological and/or pathological apoptosis, the mechanism of Hrk-mediated apoptosis has been poorly investigated. Although recent reports indicated that transcription of the *hrk* gene is under the control of the c-Jun N-terminal kinase pathway[12] as well as a transcriptional repressor DREAM[17] and that Hrk-induced apoptosis requires the expression of Bax,[12] the molecular mechanisms involved in the regulation of Hrk-mediated apoptosis still remain largely obscure.

In this study, in an attempt to identify cellular proteins that are involved in the regulation of Hrk-mediated apoptosis, we carried out yeast two-hybrid screening and isolated p32 as a novel, Hrk-interacting protein. p32 is a homotrimeric protein localized predominantly in mitochondria, whose physiological function in mammalian mitochondria remains unknown.[18,19] Functional analyses of Hrk and p32 revealed that Hrk is targeted to mitochondria to interact with p32 and that interaction with a functional p32 trimer is essential for Hrk to exert its proapoptotic activity. Thus, our data suggest a critical role for p32 in Hrk-mediated apoptosis.

Results

Isolation of p32 as an Hrk-interacting cellular factor by yeast two-hybrid screening

To identify cellular proteins that interact with Hrk, we conducted yeast two-hybrid screening of a human placenta cDNA library using Hrk as a bait. Screening of $\sim 6.4 \times 10^6$ cDNA clones yielded 24 positives, 12 of which proved to activate the reporter genes in an Hrk-dependent manner (true-positives). One of the 12 true-positives encoded Bcl-xL, an antiapoptotic Bcl-2 family member previously shown to bind Hrk,[10] and four other clones encoded Mcl-1, another antiapoptotic member expected to interact with Hrk similarly to Bcl-xL. These results underscored the idea that antiapoptotic members are interacting partners for BH3-only members,[20] and also suggested that the screening system worked properly. Interestingly, one of the remaining clones contained the entire coding region for p32,[18,21] in frame to the GAL4 activation domain (GAL4AD). The specificity of the interaction between p32 and Hrk in yeast cells was further confirmed by reintroducing the GAL4AD-p32 expression vector (pACT2-p32) into yeast cells together with expression vectors producing GAL4 DNA-binding domain (GAL4DBD) alone (pGBT9) or GAL4DBD fused to irrelevant proteins such as p53 (pVA3) and lamin C (pLAM5′) (Figure 1). p32 is a mitochondrial protein whose physiological function remains unknown, but recent crystal structure analysis suggested a possible role for p32 in apoptosis regulation.[19] We therefore characterized p32 further in this study as a possible cellular factor that interacts with Hrk both physically and functionally.

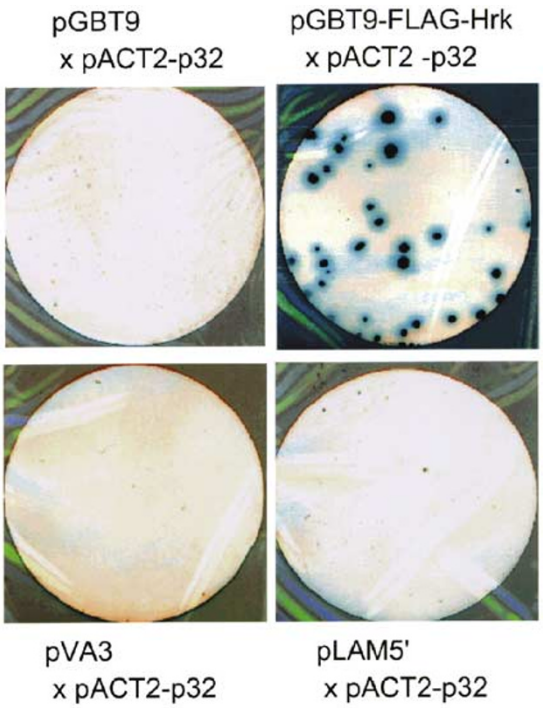

Figure 1 Specific interaction between Hrk and p32 in yeast. Yeast cells were transformed with the pACT2-p32 plasmid encoding p32 fused to the GAL4 activation domain together with a plasmid encoding either the GAL4DBD alone (pGBT9) or GAL4DBD fused to FLAG-Hrk (pGBT9-FLAG-Hrk), mouse p53 (pVA3), or human lamin C (pLAM5′). Growth of yeast in the absence of histidine and positive X-Gal staining is indicative of protein–protein interaction

Binding of Hrk and p32 *in vitro* and in mammalian cells

We further tested the specific interaction between Hrk and p32 observed in the yeast two-hybrid system through independent approaches. First, the entire coding region of p32 cDNA was transcribed and translated *in vitro*, and the *in vitro* translation product was tested for its ability to bind to the Hrk protein fused to glutathione *S*-transferase (GST). The full-length p32 protein efficiently bound to GST-FLAG-Hrk but not to GST alone, suggesting that p32 binds Hrk *in vitro* (Figure 2a). Since p32 is a highly acidic protein with pI 4.2, it was suggested that p32 may interact nonspecifically with proteins rich in basic residues, for instance, with the basic region of nuclear proteins.[18,22] To test this possibility, we included Pim-1, Cdc25A, and c-Myc (full-length and an N-terminal 262 amino-acid (a.a.) fragment that does not contain the basic region) fused to GST, all of which contain a region rich in basic residues, as controls of the binding assay. However, p32 did not bind to any of these proteins under the assay condition, underscoring the specificity of the binding (Figure 2a). We next examined whether Hrk expressed in mammalian cells

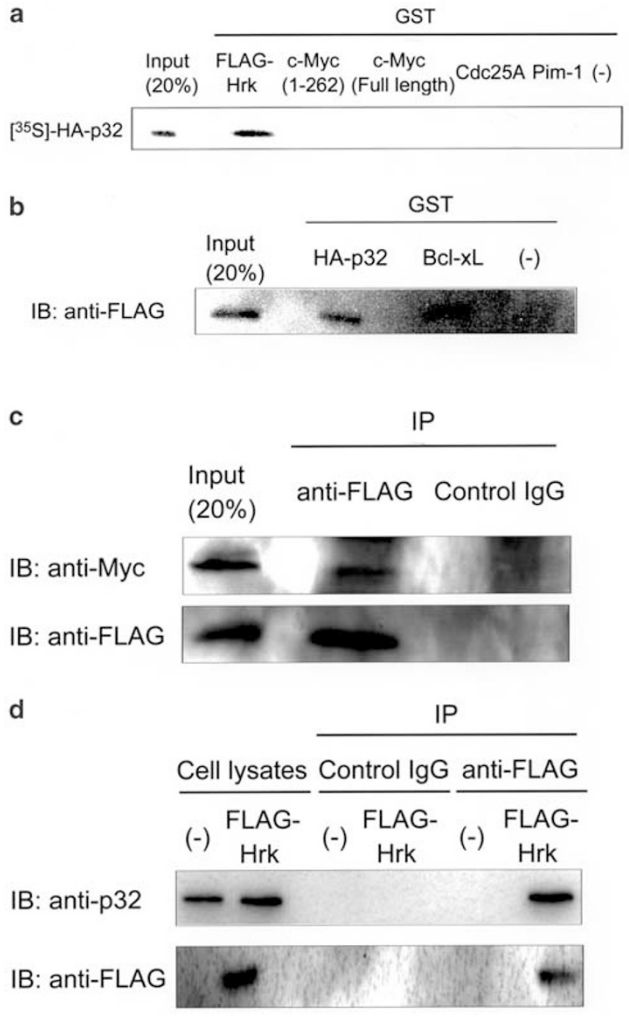

Figure 2 Specific interaction between Hrk and p32 *in vitro* and in mammalian cells. (**a**) [^{35}S]-labeled p32 protein produced by *in vitro* transcription and translation was pulled down with the indicated GST fusion proteins and detected by autoradiography after SDS-PAGE. (**b**) COS-7 cell lysates transiently transfected with a plasmid expressing FLAG-Hrk were subjected to pull-down assay with the indicated GST fusion proteins, and bound proteins were analyzed by SDS-PAGE followed by IB analysis using an anti-FLAG antibody. (**c** and **d**) COS-7 cell lysates transiently cotransfected with plasmids expressing FLAG-Hrk and p32(1-282)-Myc (**c**) or transfected with the FLAG-Hrk expression plasmid alone (**d**) were IP with an anti-FLAG antibody or with a control IgG, and the immunoprecipitates were subjected to IB analysis with anti-c-Myc (**c**) or anti-p32 (**d**), as well as with anti-FLAG antibodies. The p32 protein detected by the anti-Myc antibody likely corresponds to a mature (i.e. N-terminally processed) p32, since this band shows a migration pattern identical with that of p32(74-282)-Myc (see Figure 4)

could bind to p32 fused to GST. For this, FLAG-tagged Hrk was expressed in COS cells, and the cell lysates were incubated with GST alone or with GST fused to Bcl-xL or to full-length p32. As shown in Figure 2b, GST-HA-p32 and GST-Bcl-xL, but not GST alone, pulled down comparable amounts of FLAG-Hrk, suggesting that p32 binds Hrk as efficiently as Bcl-xL. Thus, the results of the *in vitro* binding analyses provided clear evidence that Hrk binds p32 in a specific manner. We then asked whether Hrk interacts with p32 in mammalian cell lysates. COS cell lysates expressing both FLAG-Hrk and the full-length p32 tagged with an Myc epitope at the C-terminus (p32(1–282)) were subjected to immunoprecipitation by an anti-FLAG antibody as well as by a control antibody, and the immunoprecipitates were analyzed by immunoblotting with an anti-Myc tag antibody. As shown in Figure 2c, p32-Myc was co-precipitated with FLAG-Hrk by the anti-FLAG antibody but not by the control antibody. We further wished to determine whether FLAG-Hrk interacts with endogenous, not exogenously overexpressed, p32 in the course of Hrk-induced apoptosis. To this end, lysates were prepared from COS cells transiently transfected with FLAG-Hrk, immunoprecipitated (IP) with the anti-FLAG and the control antibodies, and the presence of endogenous p32 in the immunoprecipitates was probed by anti-p32 antibody. The results clearly indicated that endogenous p32 is co-precipitated specifically with FLAG-Hrk (Figure 2d), suggesting that Hrk binds endogenous p32 during Hrk-induced apoptosis.

Binding of p32 and deletion mutants of Hrk

We then went on to determine the region of Hrk responsible for binding to p32. We divided Hrk into four regions (pre-BH3 region (a.a. 1–36), BH3 domain (a.a. 37–44), post-BH3 region (a.a. 45–58), and transmembrane (TM) domain (a.a. 59–91)), constructed a series of deletion mutants lacking one or more of these regions, and expressed these Hrk mutants in COS cells. We could not detect protein expression of some of the mutants, and therefore used for the subsequent binding analysis only those Hrk mutants schematically presented in Figure 3a, whose protein expression was confirmed by immunoblot (IB) analysis (Figure 3b). When these Hrk mutants were tested for binding with GST-HA-p32, GST-Bcl-xL, and control GST (fusion) proteins, the results indicated that none of the pre-BH3 region, the BH3 domain, the post-BH3 region, and the TM domain were required for binding with p32, whereas the BH3 domain was apparently essential for binding with Bcl-xL as reported previously.[10] Thus, unlike Bcl-xL, p32 binds Hrk independent of the BH3 domain, and the post-BH3 region of Hrk appears to be responsible for binding with p32.

The conserved C-terminal region of p32 is essential for binding and colocalization with Hrk

We next wished to define the region on the part of p32 involved in binding to Hrk. The N-terminal region (a.a. 1–73) of p32 contains a signal sequence that targets p32 to mitochondria and is removed from the mature p32 protein. p32 also has a C-terminal region (a.a. 222–282) highly conserved in different and distant species, namely, from yeast to human.[18,23] To examine the possible contribution of these N- and C-terminal regions to binding with Hrk, we constructed deletion mutants of p32 that lack the N-terminal signal sequence alone (p32(74–282)) or both the signal sequence and the C-terminal conserved region (p32(74–221)) (Figure 4a). Two versions (an Myc tag added either to the N-terminus or to the C-terminus) were created for each of the deletion mutants, expressed in COS cells, and tested for their ability to bind Hrk.

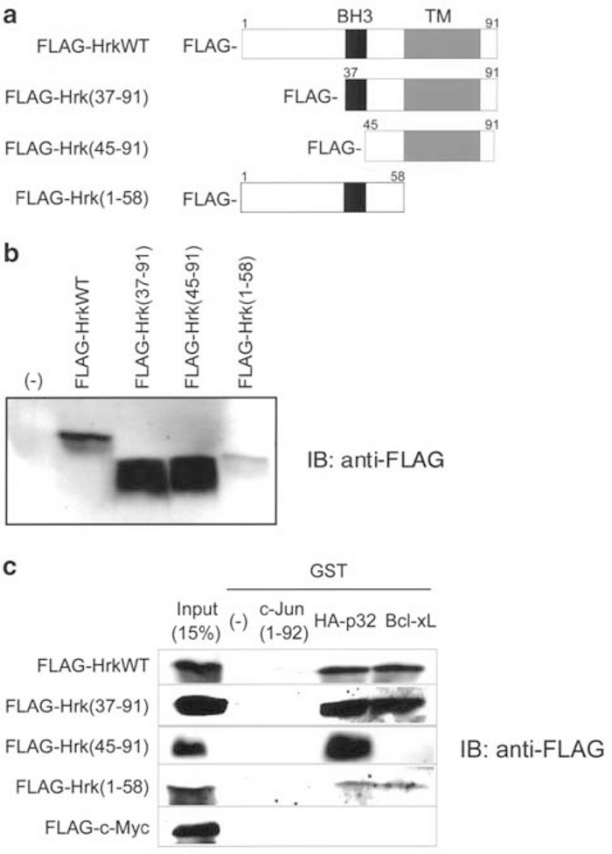

Figure 3 Binding of p32 and deletion mutants of Hrk. (**a** and **b**) Schematic presentation (**a**) and steady-state protein expression (**b**) of wild-type and deletion mutants of Hrk. (**c**) COS-7 cells were transiently transfected with plasmids expressing the Hrk constructs, and the cell lysates were subjected to pull-down assay with the indicated GST fusion proteins. Bound Hrk proteins were detected by IB analysis with an anti-FLAG antibody

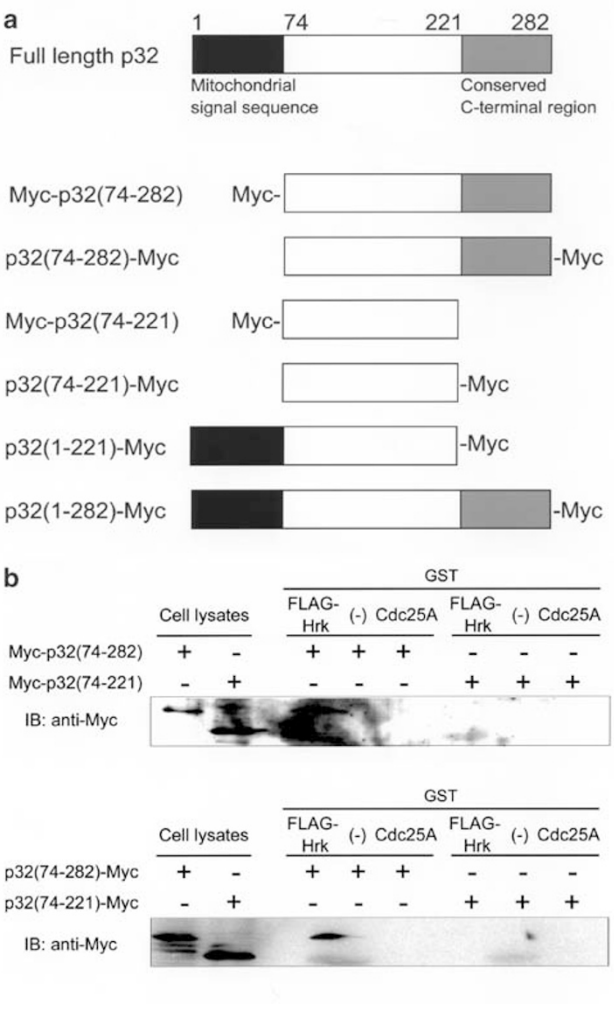

Figure 4 Conserved C-terminal region of p32 is required for binding to Hrk. (**a**) Schematic presentation of the p32 constructs used in this study. (**b**) COS-7 cells were transiently transfected with plasmids expressing p32(74–282) or p32(74–221) tagged with an Myc epitope either at the N-terminus (upper panel) or at the C-terminus (lower panel). The cell lysates were then used for pull-down assay with the indicated GST fusion proteins. Bound p32 mutants were detected by IB analysis with an anti-c-Myc antibody

As demonstrated in Figure 4b, p32(74–221) mutants were expressed at higher levels than p32(74–282), yet only p32(74–282) mutants but not p32(74–221) were pulled down by GST-FLAG-Hrk, irrespective of whether the mutants were tagged at the N-terminus or the C-terminus. Thus, Hrk binds the mature p32 protein and does not require the N-terminal signal sequence for binding. This is also supported by the results of co-immunoprecipitation assays presented earlier, since (1) the p32(1–282)-Myc protein expressed in COS cells and co-precipitated with Hrk was considered to be processed to the mature form (Figure 2c) and (2) the endogenous, mature p32 protein co-precipitated with Hrk (Figure 2d). On the other hand, the results indicated that the highly conserved C-terminal region is indispensable for p32 binding to Hrk. The attempt to examine whether the C-terminal region of p32 is 'sufficient' for binding to Hrk failed, because the C-terminal region alone appeared to be quite unstable and could not be expressed at a detectable level in mammalian cells in our hands.

We next investigated the intracellular distribution of the wild-type (p32(1–282)) and the deletion mutants of p32 by immunocytochemistry (Figure 5a). Consistent with earlier reports describing that p32 is predominantly a mitochondrial protein,[18,19,24,25] the wild-type p32 protein showed a typical pattern of mitochondrial staining. In contrast, the p32(74–282) mutant lacking the mitochondrial signal sequence, tagged either N- or C-terminally, completely lost the mitochondrial pattern and were instead localized to the nucleus as well as in the cytosol. It was also confirmed that deletion of the C-terminal conserved region has no substantial impact on the subcellular localization of p32. In parallel with p32, we also examined the subcellular distribution of Hrk. A previous study indicated that Hrk shows a 'cytoplasmic, granular distribution pattern',[10] but the precise subcellular location of Hrk remains unknown. To test the idea that Hrk is localized to mitochondria, we probed Hrk in the presence of a mitochondrial marker, MitoTracker, in the immunocytochemical analysis. As shown in Figure 5b, virtually all signals of Hrk-immunostaining colocalized with MitoTracker, indicating that Hrk is predominantly localized to mitochondria.

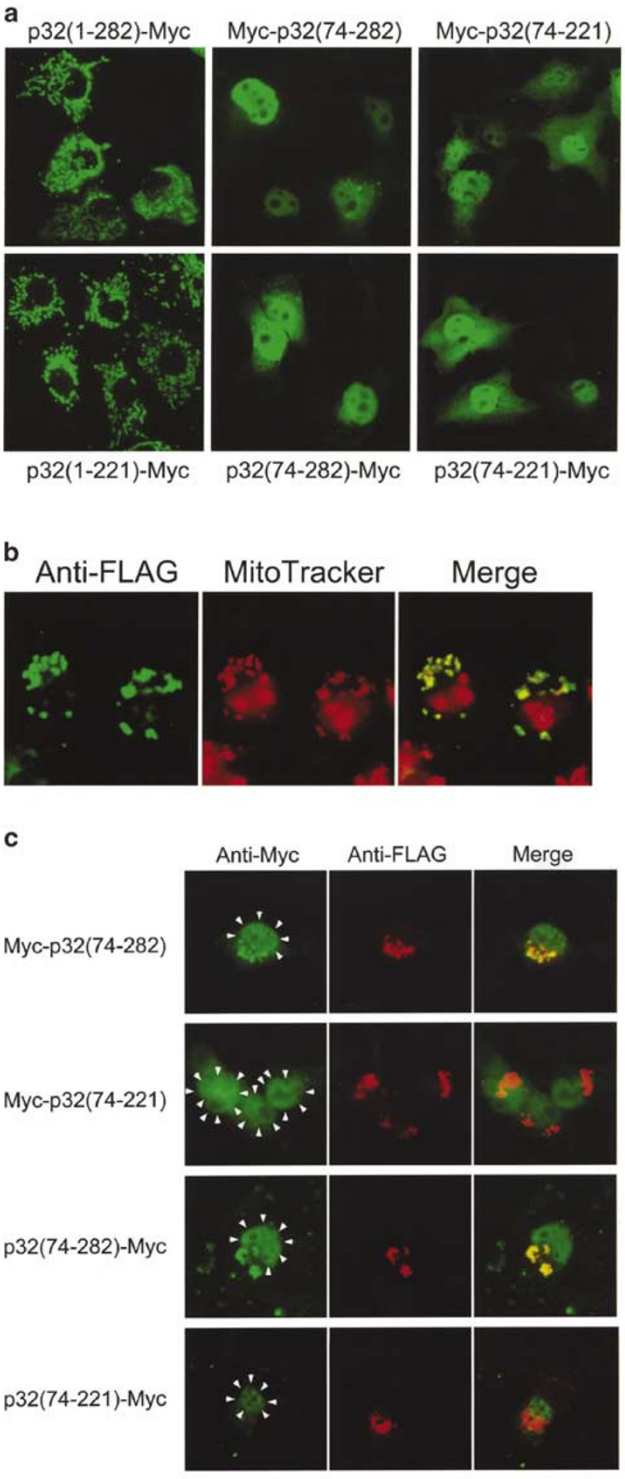

Figure 5 Subcellular localization of p32 and Hrk. (**a**) COS-7 cells were transiently transfected with plasmids expressing the indicated p32 constructs, and processed for immunofluorescence analysis using an anti-c-Myc antibody. (**b**) Hrk is targeted to mitochondria. COS-7 cells were transiently transfected with a plasmid expressing FLAG-tagged Hrk and double stained for FLAG-Hrk (green fluorescence) and mitochondria (MitoTracker, red fluorescence). (**c**) The conserved C-terminal region of p32 is essential for the colocalization of Hrk and p32. COS-7 cells were transiently cotransfected with plasmids encoding FLAG-Hrk and Myc-tagged p32 mutants and processed for immunofluorescence analysis using anti-c-Myc (green) and anti-FLAG (red) antibodies. The arrowheads indicate nuclear margin

The result that both p32 and Hrk, even when expressed independently from each other, are localized in mitochondria strongly suggested that these proteins interact and form a complex in this organelle within intact cells. We then investigated the effect of Hrk expression on the subcellular distribution of the p32 mutants, which show apparently different subcellular localization from that of Hrk when expressed separately. When expressed alone, p32(74–282) shows diffuse cytosolic and nuclear pattern as described above (Figure 5a). However, when expressed together with Hrk, a significant proportion of cytosolic p32(74–282) accumulated in a granular pattern and colocalized with Hrk (Figure 5c). Importantly, p32(74–221), which we showed to be incapable of binding Hrk, remained diffusely distributed and did not colocalize even when coexpressed with Hrk (Figure 5c). Thus, the results provide unequivocal evidence that p32 and Hrk associate within intact mammalian cells and that this association requires the highly conserved C-terminal region of p32.

Inhibition of Hrk-induced apoptosis by p32 deletion mutants lacking the N-terminal mitochondrial signal sequence

The above results suggest that the p32(74–282) mutants localized in the cytosol, but not p32(74–221) mutants incapable of binding Hrk, can compete for Hrk with the endogenous p32 protein localized within mitochondria. So, if interaction with the endogenous p32 is an integral step for Hrk to induce apoptosis, then the p32(74–282) mutants would inhibit Hrk-induced apoptosis in a dominant-negative manner. To test this possibility, we examined the effect of p32(74–282) expression on Hrk-induced apoptosis (Figure 6). We first confirmed that transfection-mediated expression of FLAG-tagged Hrk efficiently induces apoptosis in COS cells, consistent with earlier reports.[9,10] Expression of p32(74–282)-Myc or p32(74–221)-Myc did not have significant effects on the basal level of apoptosis. However, when these mutants were coexpressed together with FLAG-Hrk, FLAG-Hrk-induced apoptosis was significantly reduced by p32(74–282)-Myc but not by p32(74–221)-Myc (Figure 6a). It should be noted that, in the presence of p32(74–282)-Myc, the expression level of FLAG-Hrk was reproducibly higher than when expressed in its absence (Figure 6b). Since Bcl-xL and a pancaspase inhibitor zVAD-fmk also showed a similar effect on FLAG-Hrk expression (J Sunayama and C Kitanaka, unpublished observation), the Hrk protein may be degraded by an as yet unknown mechanism activated during apoptosis and, conversely, apoptosis inhibition may stabilize and increase Hrk protein expression. We also noticed that the FLAG-Hrk expression level tended to be lower when expressed together with p32(74–221)-Myc, for which we do not currently have an explanation (Figure 6b). The inhibitory effect of p32(74–282) was further confirmed using a different system. Since the expression of FLAG-Hrk efficiently suppressed colony formation of U251 human glioma cells, we used this system and asked whether p32(74–282) could prevent Hrk-mediated suppression of colony formation. The results shown in Figure 6c indicate that p32(74–282)

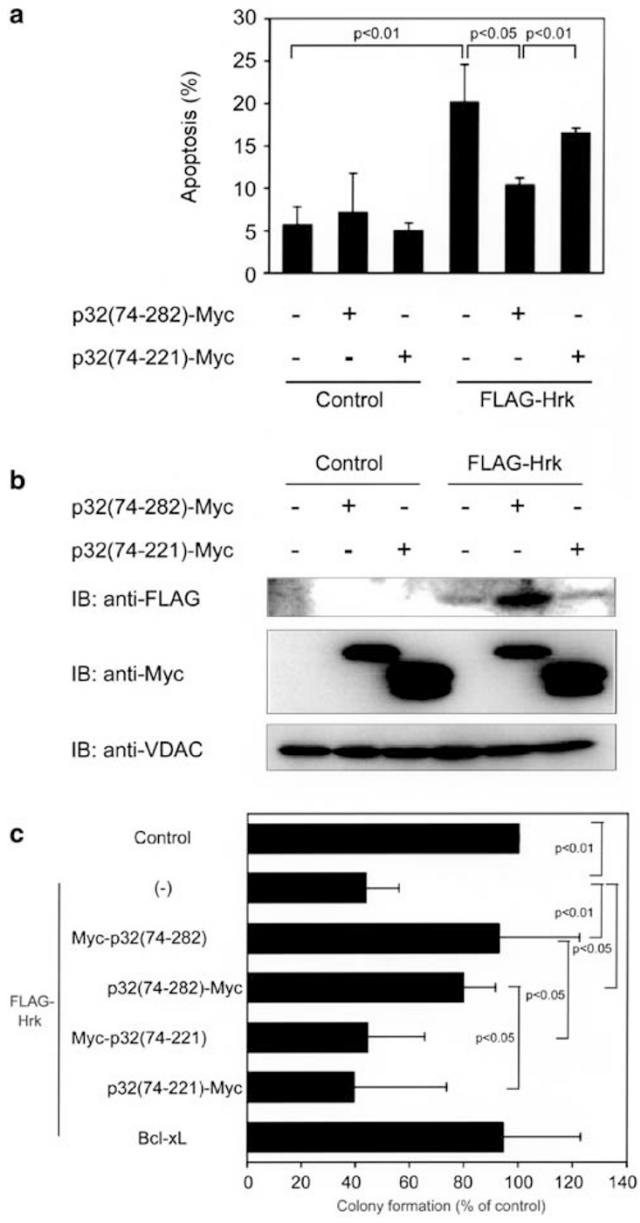

Figure 6 Inhibition of Hrk-induced apoptosis by p32 mutants lacking the N-terminal mitochondrial signal sequence. (**a** and **b**) COS-1 cells were transiently cotransfected with the GFP plasmid together with the indicated combinations of expression plasmids. (**a**) Cells were stained with Hoechst 33342, and the percentage of GFP-positive cells having apoptotic nuclei was determined. The graph shows the mean values ± S.D.s of three independent experiments. (**b**) Cells were subjected to IB analysis using anti-FLAG, anti-c-Myc, and anti-VDAC antibodies. (**c**) U251 human glioma cells were cotransfected with the pCEP4 vector plasmid to confer drug resistance and with combinations of plasmids expressing the indicated constructs. Cells were selected against the selection drug, and the number of surviving colonies was scored 10 days after transfection. The values in the graph represent $(CN/CN_{control}) \times 100(\%)$, where CN stands for the number of surviving colonies. The mean values ± S.D.s of three independent experiments are shown

constructs restored colony formation suppressed by FLAG-Hrk expression almost as efficiently as Bcl-xL, whereas p32(74–221) constructs again had no effect on colony suppression by FLAG-Hrk. Taken together, these results demonstrate that p32(74–282) inhibits Hrk-induced apoptosis in a dominant-negative manner, and also suggest that interaction with p32 is essential for Hrk to fully exert its proapoptotic activity.

A dominant-negative p32 mutant inhibits Hrk-induced apoptosis

Given the importance of p32–Hrk interaction for Hrk-induced apoptosis, we assumed as one of the possibilities that p32 may mediate the apoptotic signals elicited by Hrk. And if so, inhibition of p32 function would compromise the proapoptotic activity of Hrk. To test this idea, we designed a mutant of p32 that is expected, based on the tertiary and quaternary structure of p32,[19] to inhibit the function of p32 as a pore-forming protein in a dominant-negative manner. This mutant, p32(1–221), lacks the highly conserved C-terminal region (Figure 4a). The crystallographic study of p32 revealed that p32 is a homotrimeric protein, and that the p32 trimer has a doughnut-shaped structure with the internal channel wall being formed by beta-sheet structures from all three subunits.[19] Since the C-terminal region is considered to be essential for the stability of the beta-sheet structure,[19] inclusion of p32(1–221) in the trimer should result in the formation of a defective pore. We confirmed by immunocytochemical and binding analyses that p32(1–221)-Myc, a p32(1–221) mutant tagged with an Myc epitope at the C-terminus, was localized properly in mitochondria in COS cells similarly to the wild-type p32 protein (Figure 5a) and that p32(1–221)-Myc retains the ability to bind to wild-type p32, presumably through the N-terminus of the mature protein (Figure 7a). Both these findings ensured that the p32(1–221) mutant can associate with the endogenous p32 protein in mitochondria to form defective trimers, which is essential for the mutant to interfere with the function of endogenous p32. We then went on to test the effect of this mutant on Hrk-induced apoptosis. As shown in Figure 7b, the expression of p32(1–221)-Myc significantly reduced apoptosis induced by FLAG-Hrk. It should be noted here again that the expression level of FLAG-Hrk was reproducibly increased in the presence of p32(1–221)-Myc, which may also be a reflection of apoptosis inhibition by p32(1–221)-Myc (Figure 7c). The results of analysis using this dominant-negative p32 mutant suggest that the function of p32 as a pore-forming protein may have a significant role in mediating apoptotic signals triggered by Hrk.

Small interfering RNA (siRNA)-mediated knockdown of p32 confers protection against Hrk-induced apoptosis

To further substantiate the above finding that p32 function plays an important role in Hrk-induced apoptosis, we examined the effect of reducing p32 expression on apoptosis induction by Hrk. To this end, we utilized siRNA technique and designed an siRNA against p32. Transfection-mediated introduction of this p32-specific siRNA and a negative control RNA oligonucleotide duplexes into COS cells revealed that the anti-p32 siRNA successfully reduces the expression of the

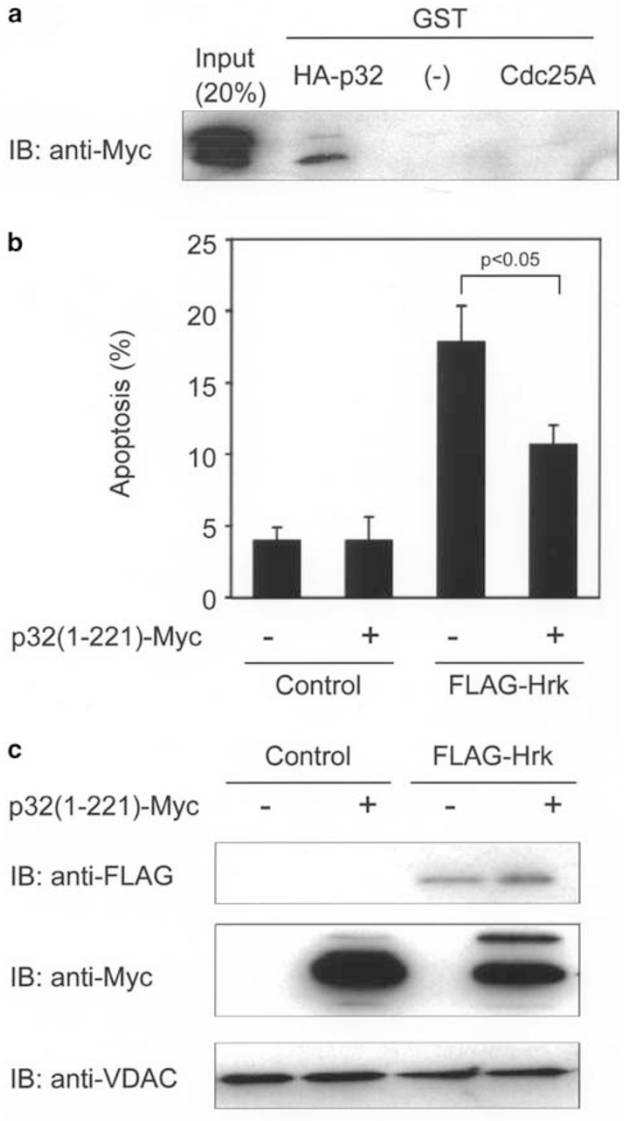

Figure 7 Inhibition of Hrk-induced apoptosis by a p32 mutant lacking the conserved C-terminal region. (**a**) p32(1–221), a p32 mutant lacking the conserved C-terminal region, retains the ability to form complex with the wild-type p32 protein. Lysates prepared from COS-7 cells transiently transfected with an expression plasmid encoding p32(1–221)-Myc were subjected to pull-down assay using the indicated GST fusion proteins. Bound proteins were analyzed by immunoblotting with an anti-c-Myc antibody. (**b** and **c**) COS-1 cells were transiently cotransfected with plasmids expressing GFP and the indicated constructs. (**b**) Cells were assayed for apoptosis, and the results represent the mean values ± S.D.s from three independent experiments. (**c**) Cells were subjected to IB analysis with anti-FLAG, anti-c-Myc, and anti-VDAC antibodies

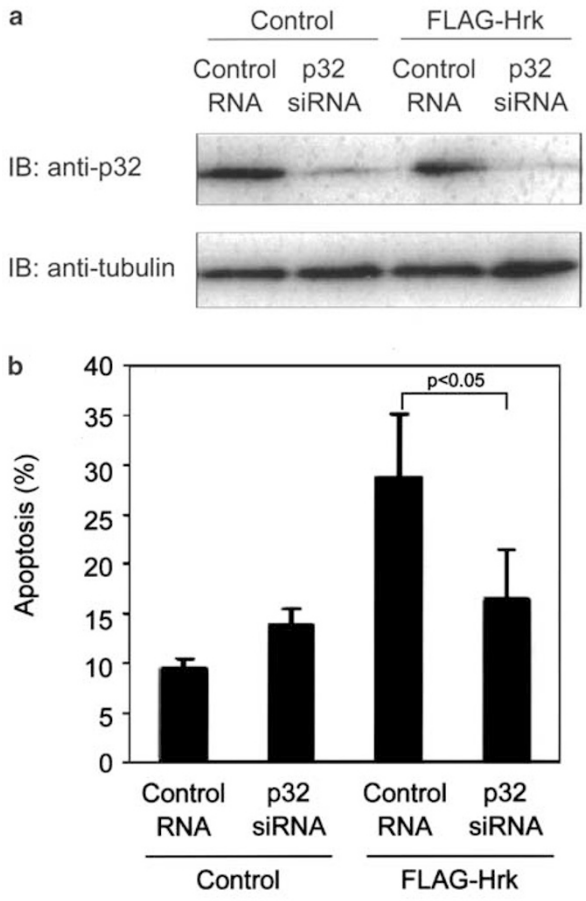

Figure 8 siRNA-mediated knockdown of p32 confers protection against Hrk-induced apoptosis. (**a** and **b**) COS-1 cells treated with anti-p32 siRNA or control RNA were transiently cotransfected with the GFP expression plasmid together with a control or FLAG-Hrk-expressing plasmid. The cells were then subjected to IB analysis to examine the expression level of the p32 protein (**a**) or to apoptosis assay (**b**). The data in the graph represent the mean values ± S.D.s from three independent experiments

endogenous p32 protein (Figure 8a). We then examined whether the siRNA-mediated knockdown of p32 protects COS cells from Hrk-induced apoptosis. As shown in Figure 8b, COS cells transfected with the anti-p32 siRNA became resistant to Hrk-induced apoptosis compared to contol RNA-transfected cells, indicating that the endogenous level of p32 expression is required for efficient induction of apoptosis by Hrk.

Discussion

In this study, we searched for cellular factors interacting with Hrk, in an attempt to elucidate the molecular mechanisms involved in Hrk-mediated apoptosis. We found by yeast two-hybrid screening that a pore-forming mitochondrial protein p32 interacts with Hrk, and specific interaction between p32 and Hrk was confirmed *in vitro* as well as in intact mammalian cells. Functional analyses using dominant-negative mutants of p32 and anti-p32 siRNA demonstrated that interaction with functional p32, as well as the physiological level of p32 expression, is essential for Hrk to fully exert its proapoptotic activity. These results strongly suggest that physical and functional interaction with p32 has a crucial role in Hrk-mediated apoptosis.

p32 was originally identified as a cellular protein associated with a nuclear pre-mRNA splicing factor SF2/ASF, and has been implicated in the regulation of splicing.[21,26] However, subsequent immunocytochemical and cell fractionation

studies revealed that p32 is predominantly a mitochondrial protein.[18,19,24,25] An analysis using yeast cells lacking the p32 homolog suggested that p32 may be involved in the regulation of oxidative phosphorylation.[18] However, otherwise, the physiological function(s) of mitochondrial p32 remains obscure. Although a number of viral and cellular proteins have been shown to interact with p32, these interactions shed little light on the biological functions of p32 in mitochondria.[25,27] On the other hand, crystallographic and biochemical studies suggested that p32 functions as a homotrimer associated with the inner (matrix) side of the inner membrane, and that the p32 trimer has a doughnut-shaped structure with a sizable central pore and a highly negatively charged surface on one side of the doughnut.[18,19,28] Based on these findings, it was speculated that p32 may regulate the intramitochondrial concentration of divalent cations such as Ca^{2+} and/or the permeability transition (PT) of the inner membrane in association with the PT pore complex, which further led to a proposal that p32 may thus be involved in the regulation of mitochondria-dependent apoptosis.[19] Here in this study, we have shown that Hrk is targeted to mitochondria to induce apoptosis accompanied by cytosolic release of cytochrome *c* (C Kitanaka, unpublished data) and that p32 plays a pivotal role in Hrk-induced apoptosis. These findings are just in line with the above prediction, and reveal a novel physiological function of p32 in mitochondria.

While the results of this study suggested that Hrk induces apoptosis through interaction with p32, it still remains unknown *how* Hrk induces apoptosis through interaction with p32. The simplest model that could be conceived based on currently available information would be that Hrk binding to p32 stimulates the pore function of the p32 trimer most likely through conformational change of the trimer. Hrk-mediated increase of the pore activity may then facilitate PT and apoptosis, since PT has been shown to have a causal role in various types of apoptosis.[2,29] Alternatively, increased pore activity of the p32 trimer may enhance apoptosis by stimulating oxidative phosphorylation. This is an interesting possibility because Hrk-induced apoptosis is dependent on Bax, and Bax-induced apoptosis has been shown to require oxidative phosphorylation in intact yeast cells[30] as well as in isolated mammalian mitochondria.[31]

It has been well established that the BH3 domain is essential for the proapoptotic activity of BH3-only members including Hrk.[10,32] Consistently, we confirmed that the Hrk mutant lacking the BH3 domain (Hrk(45–91)) does not have the ability to induce apoptosis in COS cells. However, rather unexpectedly, the Hrk mutant lacking the pre-BH3 region alone (Hrk(37–91)), which still bound Bcl-xL and p32 efficiently (Figure 3c), also failed to induce apoptosis, whereas the Hrk mutant without the TM domain (Hrk(1–58)) retained proapoptotic activity (Y Ando and C Kitanaka, unpublished observation). These findings suggest that binding to p32 alone is not sufficient for apoptosis induction, and that the entire Hrk except for the TM domain is essential for the proapoptotic activity of Hrk. Possibly, this could be explained in the following ways. First, the post-BH3 region may be required solely for complex formation by Hrk and p32, and the BH3 domain (together with the pre-BH3 region) may play an essential role in the activation of the p32 trimer. More simply, the intact BH3 domain may be required for the overall integrity of the Hrk protein to induce conformational change of the trimer upon binding. It is also possible that the BH3 domain (and the pre-BH3 region) contributes to apoptosis induction separately from p32 activation. For instance, the BH3 domain may bind and inactivate Bcl-xL and Bcl-2, and by doing so ensure that p32 activation results in efficient apoptosis induction. Intriguingly, a recent report indicated that a peptide derived from the BH3 domain of Bad is incapable of inducing apoptosis on its own, but can sensitize cells to apoptosis by occupying Bcl-xL and/or Bcl-2 in a competitive manner and increasing the intracellular concentration of 'free tBid'.[33] So, it could be envisaged that Hrk, through its BH3 domain, first frees endogenous tBid present in a very small amount and sequestered by the antiapoptotic members, and then activate p32 so that the very small amount of free tBid, which would not be sufficient to trigger apoptosis in the absence of p32 activation, could activate the multidomain members and induce apoptosis efficiently. The role of the pre-BH3 region in this scenario remains quite speculative, but given the observation that the pre-BH3 region was required for Hrk-induced apoptosis but not for *in vitro* binding with Bcl-xL and p32, this region may have an essential role *in vivo* in proper targeting of Hrk to the p32 trimer presumed to be located on the matrix side of the inner membrane.

Our data suggested that Hrk binding to p32, which occurs independently of the BH3 domain of Hrk, is a critical event in Hrk-induced apoptosis. We also found that binding to Bcl-xL via the BH3 domain is not sufficient for Hrk to induce apoptosis. These findings are consistent with the results from a structure–function study of another BH3-only member, Bik.[34] The study demonstrated that an intact BH3 domain of Bik is essential for binding to Bcl-xL as well as for its proapoptotic activity, but that neither an intact BH3 domain nor binding to Bcl-xL alone is sufficient for apoptosis induction. Interestingly, a sequence motif located within the region between the BH3 and the TM domains ('the post-BH3 region') was required for efficient apoptosis induction by Bik,[34] suggesting that requirement for another functional region outside the BH3 domain may be a common feature of BH3-only members. However, it should also be noted that the post-BH3 domains of Hrk and Bik do not share apparent homology, and we could not detect physical interaction between Bik and p32 (J Sunayama and C Kitanaka, unpublished data). So, Bik presumably has its own cofactor distinct from p32 that facilitates BH3 domain-dependent apoptosis. Similarly, we failed to detect interaction between p32 and other BH3-only members including Bid and Bad. Although it has been demonstrated that Bcl-2 can be localized in the inner mitochondrial membrane[35,36] and that Bax moves to the inner membrane during apoptosis to interact with the adenine nucleotide translocator,[37,38] presumably, most of the Bcl-2 family members may not be small enough to pass the outer membrane freely. In contrast, Hrk consists of only $\sim$50 a.a. residues outside the BH3 domain and the TM domain, making this protein one of the smallest of the proapoptotic Bcl-2 family members. Thus, Hrk may take advantage of its small molecular size and gain easier access to the inner membrane, making p32 rather a unique target of Hrk.

In summary, we have identified in this study p32 as a mitochondrial factor interacting with Hrk and demonstrated that Hrk requires interaction with functional p32 for efficient induction of apoptosis. That the BH3 domain alone (or the ability to bind Bcl-xL alone) was not sufficient for Hrk-induced apoptosis, together with the finding that interaction with p32 occurred independently from the BH3 domain, suggested a previously unrecognized mechanism for apoptosis induction by BH3-only proteins. Thus, elucidating how p32 activates the downstream apoptotic events in synergy with the BH3 domain-mediated apoptotic signal is expected to provide useful insights not only into the mechanism of Hrk-mediated apoptosis but also into the mechanism(s) by which other BH3-only proteins activate the mitochondrial step of apoptosis.

Materials and Methods

Plasmid construction

pcDNA3-FLAG-Hrk is a gift from Dr. Naohiro Inohara (The University of Michigan Medical School) and has been described previously.[10] The FLAG-tagged Hrk cDNA fragment was excised from pcDNA3-FLAG-Hrk and subcloned into pGBT9 (Clontech) in frame to the GAL4DBD and into pGEX2T in frame to GST, to create pGBT9-FLAG-Hrk and pGEX2T-FLAG-Hrk, respectively. pACT2-p32 was originally isolated in this study from a human placenta cDNA library by two-hybrid screening, and encodes a p32 protein lacking the first three a.a. residues and tagged with a hemagglutinin (HA) epitope at the N-terminus. For construction of pGEX2T-HA-p32 and pcDNA3-HA-p32, the HA-tagged p32 cDNA fragment was excised from pACT2-p32 and subcloned into pGEX2T vector in frame to GST and into pcDNA3, respectively. cDNA fragments of full-length (p32(1–282)) as well as deletion mutants of p32 (p32(1–221), p32(74–282), p32(74–221)) were amplified by PCR using pACT2-p32 as a template and subcloned into pcDNA3.1-Myc-His B vector (Invitrogen) and to pc3GF-Myc vector[39] to provide a C-terminal and an N-terminal Myc tag, respectively. Similarly, wild-type and deletion mutants of Hrk were amplified by PCR using pcDNA3-FLAG-Hrk as a template and subcloned into pFLAG CMV2 vector (Kodak) in frame to the FLAG epitope. All PCR-generated fragments were sequenced for verification. pGEX2T-c-Myc(1–262), pGEX2T-Pim1, and pGEX2T-Cdc25A, pGEX4T-c-Jun(1–92), pcDNA3EGFP have been described previously.[39–41] pGEX2T-c-Myc (full-length) was constructed by subcloning into pGEX2T a cDNA fragment containing the entire coding region of human c-myc in frame to GST. pcDNA3-mBcl-xL and pGEX2T-mBcl-xL are gifts from Dr. Yoshihide Tsujimoto (Osaka University Medical School) and express mouse Bcl-xL in mammalian cells and mouse Bcl-xL fused to GST in *Escherichia coli*, respectively. pVA3 and pLAM5′ were purchased from Clontech.

Yeast two-hybrid screening

A human placenta cDNA library fused to the GAL4AD of the pACT2 vector (Clontech) was screened for proteins that interact with human Hrk, using the Y153 yeast strain. Briefly, the pACT2 library plasmid was transformed into Y153 yeast cells harboring the pGBT9-FLAG-Hrk bait plasmid by standard transfection procedures. Transformed Y153 cells were plated on medium lacking tryptophan, leucine, and histidine containing 3-amino-1,2,4-triazole. A total of 6.4×10^6 library clones were screened for growth in selection medium and assayed for β-galactosidase activity. pACT2 plasmids were recovered by bacterial transformation of plasmids isolated from positive yeast colonies. The candidate pACT2 cDNA plasmids were retransformed into yeast cells with the empty pGBT9 or pGBT9 plasmids encoding irrelevant baits to exclude false-positives. Inserts of true-positive pACT2 cDNA clones were characterized by restriction enzyme mapping and nucleotide sequence analysis using an automated DNA sequencer.

Mammalian cell culture and transfection

COS-1, -7 cells and U251 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 1% penicillin/ streptomycin (Gibco). COS-7 and U251 cells were transfected by the use of Effectene (Qiagen) and COS-1 cells by Fugene (Roche). Unless otherwise indicated, transfected cells were subjected to analyses on the next day of transfection. In principle, transfection of Hrk-expressing plasmids for binding and immunofluorescence analyses was carried out in the presence of zVAD-fmk to enhance the expression level of Hrk proteins and to prevent detachment of apoptotic cells, respectively.

GST pull-down assay

GST fusion proteins were produced from pGEX2T-based vectors in *E. coli* JM109 by the addition of isopropyl-β-thiogalactopyranoside and purified with glutathione–sepharose 4B beads (Amersham). *In vitro* transcription-coupled translation (Promega) was carried out using a linearized pcDNA3-HA-p32 plasmid as a template and in the presence of [^{35}S]methionine according to the manufacturer's protocol. COS cell lysates expressing various Hrk or p32 constructs were obtained by lysing cells in the lysis buffer (20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA (pH 8.0), 0.2% NP-40). Pull-down assays were performed by incubating the *in vitro* translation product or the cell lysates with GST fusion proteins immobilized on the glutathione beads for 6 h at 4°C. Protein complexes captured on the beads were eluted in the SDS sample buffer, separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and subjected to IB or autoradiographic analyses.

Co-immunoprecitation assay

Cells were lysed in the lysis buffer (20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA (pH 8.0), 0.2% NP-40), and the lysates precleared with protein A–Sepharose beads (Amersham) were incubated with an anti-FLAG mouse monoclonal antibody (M5, Kodak) or with a control mouse IgG at 4°C for 5 h, and subsequently with protein A–Sepharose beads for 1 h. The protein–antibody complex recovered on the beads was subjected to IB analysis after separation by SDS-PAGE.

IB analysis

Cells were lysed in the lysis buffer (20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA (pH 8.0), 0.2% NP-40), and after determination of protein concentration using a BCA protein assay kit (Pierce), equal amounts of protein samples were resolved in a SDS-PAGE and electrically transferred to a nitrocellulose membrane. The membrane was probed with an appropriate primary antibody (anti-FLAG (M5, Kodak), anti-c-Myc (Ab-1, Calbiochem), anti-voltage-dependent anion channel (VDAC) (31HL, Calbiochem), anti-α-tubulin (DM1A, Sigma), and anti-p32 (Muta *et al.*[18])), and then with a horseradish peroxidase-conjugated secondary antibody. Blots were visualized by enhanced chemiluminescence.

Immunofluorescence analysis

COS cells were grown on glass coverslips and transfected with a plasmid expressing FLAG-tagged Hrk and/or with expression plasmids encoding Myc-tagged p32 proteins. Cells were fixed and permeabilized in phosphate-buffered saline (PBS) containing 4% paraformaldehyde and 0.3% Triton X-100 for 15 min, followed by blocking in PBS containing 2% bovine serum albumin (BSA) and 0.3% Triton X-100 for 15 min. Cells were then incubated with an anti-FLAG antibody (M5, Kodak) and an anti-Myc tag polyclonal antibody (Cell Signaling Technology) in Tris-buffered saline for 4 h at 37°C. After subsequent incubation with rhodamine- and fluorescein isothiocyanate-conjugated secondary antibodies in the blocking buffer for 1 h, cells on the coverslips were mounted on glass slides and observed under a fluorescence microscope. Where indicated, cells were stained with MitoTracker Orange (Molecular Probes) prior to fixation to visualize mitochondria.

Apoptosis assay

pcDNA3EGFP was cotransfected into COS cells together with expression plasmids encoding Hrk and various p32 proteins. Cells were stained with Hoechst 33342 for 10 min, and the percentage of green fluorescent protein (GFP)-positive cells with apoptotic (condensed and/or fragmented) nuclei was determined by Laser Scanning Cytometer (OLYMPUS).[42]

Colony formation assay

U251 cells were cotransfected with pCEP4 (carrying a hygromycin-resistance gene) together with expression plasmids encoding Hrk, Bcl-xL, and various p32 proteins. Transfected cells were selected against hygromycin B, and the number of surviving colonies was scored 10 days later.

siRNA

siRNA oligonucleotide duplexes were obtained from FASMAC in the annealed and purified form. The sense sequences of the anti-p32 siRNA and the control small RNA were 5′-GGUUGAAGAACAGGAGCCUdTdT-3′ (corresponding to nucleotides 462–480 of the coding region of human p32) and 5′-AGGCCGACAUGAGGUAGAUdTdT-3′ (a scrambled sequence of the anti-p32 siRNA), respectively. In the design of the siRNA, a National Center for Biotechnology Information database BLAST search was performed to ensure target specificity. Transfections of RNA duplexes were carried out using Oligofectamine reagent (Invitrogen). In brief, 3 μl of Oligofectamine reagent with 100 pmol RNA oligonucleotide duplexes were used per well (six-well dish). Cells were subjected to further analyses 48 h after siRNA transfection.

Statistical analysis

Results were expressed as means $\pm$ standard deviations (S.D.) and analyzed using the unpaired Student's *t*-test.

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