c-Myc Augments the Apoptotic Activity of Cytosolic Death Receptor Signaling Proteins by Engaging the Mitochondrial Apoptotic Pathway

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Activation of c-Myc sensitizes cells to apoptosis induction by ligand-activated death receptors. Such sensitization to death receptors by oncogenes may well be the mechanism underlying tumor cell sensitivity to tumor necrosis factor (TNF) or TNF-related apoptosis-inducing ligand (TRAIL). The mechanism by which this c-Myc-induced sensitization occurs is unclear but could involve modulation of expression of death receptors or their ligands or potentiation of the sensitivity of mitochondria to release pro-apoptotic effectors such as mitochondrial cytochrome c. Here, we show that ectopic expression of the death receptor signaling protein RIP (receptor-interactive protein) triggers apoptosis via a FAS-associated death domain protein (FADD) and caspase-8-dependent pathway. Induction of apoptosis by this intracellular activation of the death receptor signaling pathway is significantly augmented by c-Myc expression. Moreover, c-Myc expression strongly promotes the potential of RIP to induce cytochrome c release from mitochondria. This implicates the mitochondrial apoptotic pathway in this synergy, a notion confirmed by the inability of c-Myc to sensitize to RIP killing in cells lacking the obligate mitochondrial apoptotic effectors Bax and Bak. We conclude that the lethality of the RIP-activated cytosolic caspase-8 pathway is augmented by c-Myc priming mitochondria to release cytochrome c. This places the intersection of apoptotic synergy between c-Myc and death receptor signaling downstream of the death receptors.

Deregulated expression of the c-Myc oncoprotein causes uncontrolled cell proliferation. The immediate consequence of such proliferative deregulation, which characterizes most, if not all, human cancer cells (1) can be either cell expansion or, paradoxically, the destruction of affected cells through apoptosis. Both cell culture and animal models have shown that the ultimate fate of a cell population expressing activated c-Myc depends on cell type and the availability of survival factors as well as on the presence of any anti-apoptotic lesions (for review, see Ref. 2). Understanding how to favor the apoptotic aspect of c-Myc over its proliferative functions offers the potential for selective ablation of tumor cells.

In cultured cells, the presence of survival factors such as insulin-like growth factor-1 or serum counteracts the apoptotic action of c-Myc (3). However, even in the presence of such survival factors, cells with deregulated c-Myc remain hypersensitive to multiple apoptotic insults including genotoxic damage, hypoxia, interferon-γ, irradiation, and NK cells (Ref. 2 and references therein). Furthermore, both c-Myc and the Rh pathway effector E2F-1 can induce sensitivity to ligand-induced activation of the cell surface death receptors p55 TNFR, TRAIL receptors, and CD95 (4–7). In the specific instance of TNFR, part of the increased TNF sensitivity is attributable to a c-Myc- or E2F-1-induced failure of TNFFR to activate NF-κB-dependent survival pathways (5, 8), rendering the cells more vulnerable to the apoptotic signal initiated by ligand-bound TNFR. This death signal is mediated by FADD and caspase-8, whose pro-forms are recruited to the cytosolic tail of death receptors in a death receptor-induced signaling complex (DISC) (9, 10). It is thought that in the death receptor-induced signaling complex, the close proximity of zymogenic procaspases 8 triggers auto-proteolytic activation of the pro-caspases such as caspase 3.

The ability of c-Myc to promote TNFR-initiated apoptosis as well as to induce apoptosis upon survival factor deprivation is strongly suppressed in cells overexpressing Bcl-2 (6, 11). Bcl-2 proteins counteract apoptotic sensitization by c-Myc in multiple tissues, and this is thought to be the mechanism underlying oncogenic synergy between c-Myc and Bcl-2 (12, 13). Bcl-2 belongs to the BH3 domain carrying mitochondrial Bcl-2 family proteins comprising anti- (e.g. Bcl-2 and Bcl-xL) and pro-apoptotic (e.g. Bax and Bak) members. These proteins modulate each others’ activities by forming heterodimers that regulate the release of cytochrome c (cyt c) from the mitochondrial intermembrane space into the cytosol. When the anti-apoptotic BH3 proteins dominate, cyt c release is inhibited, whereas the pro-apoptotic BH3 proteins promote cyt c release (14). In the cytosol, cyt c activates apoptosis by binding and activating the Apaf-1-caspase 9 complex. This “apoptosome” acts as a processing/activation center for the downstream caspase 3 (15). Analogous to the overexpression of anti-apoptotic Bcl-2, lack of pro-apoptotic Bax also renders cells refractory to the apoptotic

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activity of c-Myc (16). Furthermore, in a cell population deprived of survival factors, c-Myc induces a rapid cyt c release that precedes any other detectable apoptotic changes (17). Taken together, these data indicate that the c-Myc-dependent activation of the mitochondrial apoptosis pathway plays a key role in the apoptotic sensitization.

In the specific instance of TNFR, sensitization by c-Myc in part involves inhibition of the NF-κB survival pathway. However, neither CD95 nor TRAIL death receptors consistently activated the NFATAME2F-1 constructs were activated by the addition of 100 nM 4-hydroxytamoxifen (4-OHT). Rat-1-p65, and Rat-1-SRI cells were plated in 10-cm dishes instead of 6-cm dishes before the transfection. Chloroquine was omitted, and the cells were transfected using a liposome-mediated transfection kit (LipofectAMINE Plus, Invitrogen) rather than calcium chloride. Retroviral supernatants were collected, and 1:200 in the 3% bovine serum albumin-saponin buffer was then added to the cells followed by 2-h incubation. Cells were washed with phosphate-buffered saline and fixed in the wells for 15 min using 4% paraformaldehyde, washed again, and stained with 0.5% glutaraldehyde, washed, and stained with β-galactosidase-specific staining solution (8). After overnight incubation at 37°C in cells per well were examined for apoptosis by microscopic analysis. Apoptotic cells exhibited characteristic round or irregular morphology, which was easily distinguishable from the morphology of non-apoptotic fibroblasts. Transfection experiments employing a green fluorescent protein (GFP)-encoding vector pBMN-ires-GFP showed a correlation between the intensity of fluorescence in transfected cells and the amount of expression vector that was included in the transfection mixture. In flow cytometric analysis, the mean green fluorescence values (arbitrary units) obtained for pBMN-ires-GFP-transfected cells were 41, 59, 73, 85, 96, and 142 when 0.0, 0.05, 0.1, 0.2, 0.4, and 0.8 μg, respectively, of pBMN-ires-GFP was included in the transfection mixture (in each case the total plasmid concentration was balanced to 1 μg with pBabe-puro DNA). This established a correlation between the protein expression level in the individual transfected cells and the concentration of corresponding expression vector in the transfection mixture.

**Retroviral Infection of Cells**—High titer retroviral stocks were isolated from transiently transfected Phoenix-E packaging cells following the inventor’s protocol with minor changes (www.stanford.edu/group/nolan/protocols/pro_helper_free.html). Briefly, Phoenix-E cells (6 × 10^6) were pelleted 1:200 in the 3% bovine serum albumin-saponin buffer was then added to the cells followed by 2-h incubation. Cells were washed with phosphate-buffered saline and fixed in the wells for 15 min using 4% paraformaldehyde, washed again, and stained with 0.5% glutaraldehyde, washed, and stained with β-galactosidase-specific staining solution (8). After overnight incubation at 37°C in cells per well were examined for apoptosis by microscopic analysis. Apoptotic cells exhibited characteristic round or irregular morphology, which was easily distinguishable from the morphology of non-apoptotic fibroblasts. Transfection experiments employing a green fluorescent protein (GFP)-encoding vector pBMN-ires-GFP showed a correlation between the intensity of fluorescence in transfected cells and the amount of expression vector that was included in the transfection mixture. In flow cytometric analysis, the mean green fluorescence values (arbitrary units) obtained for pBMN-ires-GFP-transfected cells were 41, 59, 73, 85, 96, and 142 when 0.0, 0.05, 0.1, 0.2, 0.4, and 0.8 μg, respectively, of pBMN-ires-GFP was included in the transfection mixture (in each case the total plasmid concentration was balanced to 1 μg with pBabe-puro DNA). This established a correlation between the protein expression level in the individual transfected cells and the concentration of corresponding expression vector in the transfection mixture.

**Flow Cytometric Analysis of Apoptosis and Sorting**—For flow cytometric analysis, trypan-detached cells were transferred to 5-ml round-bottom tubes (Falcon). Cells were washed once with phosphate-buffered saline (PBS)-containing 2 mM EDTA and 200 μM transfected cells by Western blot analysis with specific antibodies. MEF-MycERTAM cells were created as previously described (8). All cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and antibiotics. The established cell lines containing expression constructs were periodically grown in selection medium. The MycERTAM and ERATAM-E2F-1 constructs were activated by the addition of 100 nM 4-hydroxytamoxifen (OHT; Sigma) to the cell culture medium if not otherwise stated in the text. Caspases were inhibited with 50 μM final concentration of zVAD-fmk (Enzyme Systems Products).

**Gene Transfer Constructs**—The RIP deletion mutants were created by PCR using murine RIP cDNA as a template. Amino acid point mutation K45R that specifically eliminates the kinase activity or RIP (22) was introduced into kinase-dead RIP (RIPKι) by using the QuikChange mutagenesis kit (Stratagene). Wild type RIP and RIP mutants were subsequently cloned into pcDNA 3.1+ (Invitrogen) and retroviral pBMN-ires-GFP expression vectors. Protein expression from these constructs was verified from total lysates of transfected or transduced cells by Western blot analysis using specific antibodies. The cDNAs of CrmA, p35, FADD, dominant-negative caspase 8, and Bcl-xL were all in CMV promoter-driven expression vectors.

**Transient Transfection of Cells**—For transfection, cells were seeded at a density of 150,000 cells/well into 6-well plates. The next day, cells were transfected with mixtures of expression plasmid DNAs using FuGENE 6 (Roche Molecular Biochemicals) reagent. Each transfection mixture contained a total amount of 1.2 μg of plasmid DNA made up from a combination of 1 μg of effector plasmid (adjusted with empty plasmid DNA as needed) and 0.2 μg of CMV-β-galactosidase. CMV-β-galactosidase was included in the mix to mark the transfected cells by β-galactosidase-specific staining. Cells were transfected for 3 h after which the lipid-DNA complexes were washed off from the cells. In experiments with Rat-1 cells carrying MycERTAM or ERATAM-E2F-1 construct, either OHT (c-Myc/E2F-1 ON) or ethanol carrier (c-Myc/E2F-1 OFF) was added to the cells at 6 h post-transfection. 24 h after transfection, cells were washed with phosphate-buffered saline, fixed with 0.05% glutaraldehyde, washed again, and stained with β-galactosidase-specific staining solution (8). After overnight incubation at 37°C, cells were pelleted 1:200 in the 3% bovine serum albumin-saponin buffer was then added to the cells followed by 2-h incubation. Cells were washed with buffer and subsequently incubated with the secondary antibody Alexa Fluor568 (Molecular Probes) for 2 h at 37°C. Before immunofluorescence microscopy, cells were washed with buffer containing Hoechst 33342 (Sigma) to stain the cell nuclei. The samples were examined with an inverted fluorescence microscope (Zeiss Axiovert 5100 TV) equipped
with appropriate filters for Alexa Fluor568, Hoechst 33,342 and GFP. Images were collected with a CCD camera (Hamamatsu Orca) and processed with OpenLab software (Improvision). Merged images were obtained with the same software.

To analyze the amount of cyt c in mitochondria-containing cell fractions, the mitochondria-containing heavy membrane fractions were essentially isolated as described in Juin et al. (17). The heavy membrane fractions, solubilized in radioimmune precipitation assay (RIPA) buffer, were examined for cyt c by Western blot analysis using a cyt c-specific antibody (Pharmingen).

**NF-κB Luciferase Assay**—To measure NF-κB activity, cells were seeded on 12-well plates and transfected as described above with plasmid mixtures, each containing a total of 1.15 µg of plasmid including 0.1 µg of NF-κB-driven plasmid pBIIIX-Luc and 0.05 µg of pRL-TK. pRL-TK directs expression of Renilla luciferase under a constitutive promoter and was used as an internal control to normalize the obtained NF-κB values. Transfected cells were lysed at 24 h post-transfection, and both the firefly and Renilla luciferase activities were measured using the dual-luciferase reporter assay kit (Promega) and automated microplate luminometer (LB96V, EG&G Berthold).

**Caspase Assays**—Cells were grown in 6-well plates and at defined time points lysed by the addition of 100 µl of lysis buffer (10 mM Tris, pH 7.5, 130 mM NaCl, 1% Triton X-100, 10 mM NaPi, 0.5 mM NaPPi) per well. Lysates were immediately chilled on ice, clarified by centrifugation, and assayed for total protein concentration. To measure caspase activities, 60–80-µg aliquots of each lysate was incubated in a 100-µl total volume of 1× Salvesen optimal reaction buffer (23) made from 5× stock and supplemented with 200 µM colorimetric peptide substrate from Calbiochem, except for Ac-VDVAD-pNA, which was from Alexis. Samples were incubated at 37 °C and read at 405 nm with an automated plate-reading spectrophotometer (Wallac) in 1-h intervals.

**RESULTS**

**c-Myc Augments the Lethality of FADD, Pro-caspase 8, and RIP**—To define whether death receptor signaling proteins co-operate with c-Myc in the induction of cell death, expression vectors encoding individual signaling proteins were introduced by transfection into Rat-1 fibroblasts containing a conditionally active, OHT-inducible form of c-Myc (MycERTAM). All studies were conducted on Rat-1 cells growing in high serum, under which conditions c-Myc deregulation alone does not induce appreciable apoptosis. The extent of death induced by pro-apoptotic proteins correlated well with the dose of plasmid offered to the cells (see white bars in Fig. 1B), suggesting that different concentrations of expression plasmid induce different levels of protein expression in individual cells. Studies using a fluorescent marker protein confirmed this by showing a correlation between the marker plasmid concentration used for transient transfection of cell pools and the average expression level of marker protein present in each transfected cell (see “Experimental Procedures”).

We observed a significant c-Myc-dependent augmentation of the death-inducing activity of FADD, pro-caspase 8 (Fig. 1A) and RIP (Fig. 1B). FADD was extremely lethal to cells but, when expressed at very low levels (10 ng of plasmid in the transfection mix), it induced only modest cell death that was significantly enhanced upon activation of c-Myc (Fig. 1A). Likewise, c-Myc also enhanced the lethality of pro-caspase 8 although less markedly than for FADD. As with FADD and pro-caspase 8, when RIP was expressed at low levels, at which it was by itself only weakly lethal, activation of c-Myc induced a significant increase in cell death (Fig. 1B). When expressed at high levels, RIP (cells transfected with >1.0 µg of expression plasmid), FADD (>0.05 µg), and pro-caspase 8 (>0.5 µg) all potently induced cell death, and this was only modestly or not at all exacerbated by c-Myc activation (data not shown). Because of the extreme lethality of FADD, we used RIP as an intracellular trigger of cell death in the following studies.

RIP is a death receptor adaptor protein with an N-terminal serine/threonine kinase domain and a C-terminal death domain (22, 24). To determine which regions of RIP trigger the death signaling pathway that is augmented by c-Myc, constructs encoding the death domain-deficient form of RIP (RIPDD), death domain of RIP alone (RIPDD), and a kinase-dead RIP (RIPKin) were each transiently transfected into MycERTAM cells, and the ensuing cell death was analyzed as above. We found that RIP requires its death domain to kill and to co-operate with c-Myc in death induction (Fig. 1C). However, the death domain on its own, although capable of inducing cell death, did not co-operate with c-Myc. The kinase activity of RIP is required for optimal death induction (Fig. 1C and Holler et al. (25)). However, the lack of kinase activity did not abolish the lethal co-operation of RIP with c-Myc. As a control for c-Myc function, we used a MycERTAM mutant Myc106–143ERAM that lacks its N-terminal transcription activation domain, were transfected with pDNA3.1-RIP and analyzed as in A. The c-Myc-dependent relative increase in the RIP killing (c-Myc OFF 100%) is shown.

**FIG. 1.** c-Myc augments the apoptotic activity of FADD, pro-caspase 8, and RIP. A, effect of c-Myc on the FADD and pro-caspase 8-induced cell death. Rat-1-MycERTAM cells were co-transfected with pCDNA3.1 vector directing expression of FADD or pro-caspase 8 and CMV-β-galactosidase. At 6 h post-transfection, c-Myc was activated by the addition of OHT to the growth medium, and cells were grown for a further 18 h, fixed, and stained for β-galactosidase. The percentage of β-galactosidase-positive cells is plotted as bars. Experiments were repeated at least three times. The statistical significance of the difference between c-Myc OFF and ON populations was assessed by two-way analysis of variance. The two asterisks denote the statistically significant difference with p value less than 0.01. B, effect of c-Myc on the RIP-induced cell death. Rat-1-MycERTAM cells were co-transfected with pCDNA3.1-RIP and CMV-β-galactosidase vectors. The assay, analysis of cell death, and statistical tests were performed as in A. C, effect of c-Myc on the lethal activity of RIP mutants. Rat-1-MycERTAM cells were co-transfected with pCDNA3.1 vectors encoding death domain-deficient RIP (RIPDD), death domain of RIP (RIPDD), or kinase-dead RIP (RIPKin) and CMV-β-galactosidase vector. D, only a transcriptionally active c-Myc augments the apoptotic activity of RIP. Rat-1 cells expressing MycERTAM or Myc106–143ERAM which lacks its N-terminal transcription activation domain, were transfected with pDNA3.1-RIP and analyzed as in A. The c-Myc-dependent relative increase in the RIP killing (c-Myc OFF 100%) is shown.

In summary, c-Myc augments the apoptotic activity of death
receptor-signaling proteins FADD, caspase 8, and RIP but only if they are expressed at a level that is only weakly apoptotic. Furthermore, in the case of RIP, the C-terminal death domain is essential, although not sufficient, to activate an apoptotic pathway that synergizes with c-Myc.

The Apoptotic Activity of RIP Requires FADD and Caspase 8—Biochemical and genetic evidence both place FADD and caspase 8 in the same death receptor pathway (10). However, much less is known of the pathways that mediate the apoptotic action of RIP. Because c-Myc sensitizes to FADD, pro-caspase 8, and RIP, one possibility is that all three lie on the same signaling pathway. To explore this possibility, we examined the dependence of RIP-induced cell death on FADD and caspase 8.

First, we confirmed that RIP-induced cell death has the hallmarks of classical apoptosis and is mediated by caspases. Rat-1 cells were infected with pBMN-RIP retroviruses that dictate the bicistronic expression of both RIP and GFP. To characterize the ensuing cell death, infected cells were stained with Annexin V, which binds to externalized phosphatidylserine on the surface of apoptotic cells, and Annexin V binding was quantitated by flow cytometry in GFP-positive cells. We found that RIP potently triggers expression of external phosphatidylserine and that this was blocked by the presence of cell-permeable pan-caspase inhibitor zVAD-fmk (Fig. 2A, left panel). Analysis of caspase activity showed that RIP potently activates DEVD-specific caspases and that zVAD-fmk completely blocks this caspase activation (Fig. 2A, right panel). Thus, RIP induces caspase-dependent apoptosis in Rat-1 cells.

We next asked if RIP-induced apoptosis and activation of DEVD-specific caspases (caspases 3 and 7) is dependent on caspase 8 using MEFs derived from caspase 8 knockout mice. We saw no induction of apoptosis by RIP in caspase 8-deficient MEFs (Fig. 2B). We then examined whether RIP induces caspase activity in caspase 8-deficient MEFs by incubating cell lysates with various colorimetric caspase substrate peptides. IETD peptide was used as a preferred substrate for caspase 8, and DEVD peptide was used as a substrate for caspase 3/7 activity (26). Compared with wild type MEFs, we saw only negligible activities against either peptide in caspase 8-deficient cells (see Fig. 2C for DEVD, data not shown for IETD). Thus, RIP requires caspase 8 to trigger apoptosis and downstream caspases.

RAIDD is an adaptor molecule with the potential to bind both RIP and caspase 2 (27), raising the possibility that RIP might activate caspase 2 independently of caspase 8. To test for RIP activation of caspase 2 we used the caspase 2-specific colorimetric substrate penta-peptide VDVAD (28). However, we saw significant cleavage of VDVA-D only in caspase 8-expressing cells (Fig. 2D). This indicates that caspase 8 is also required for the RIP activation of caspase 2-type activity.

Last, we determined the role of caspase 8 in the synergistic apoptosis induction by RIP and c-Myc. We retrovirally introduced MycERTAM into caspase 8-deficient MEFs and control cells. We found that RIP completely failed to kill cells lacking caspase 8 even if c-Myc was active (Fig. 3A). Thus, caspase 8 is essential for the lethal cooperation of RIP and c-Myc.

To explore whether RIP employs FADD-dependent or -independent mechanisms to induce caspase 8-mediated apoptosis, we functionally inhibited FADD with a dominant-negative form of FADD (10). As shown in Fig. 3B, expression of dominant-negative FADD (DNFADD) profoundly inhibits RIP-induced cell death and the synergistic death induction by RIP and c-Myc (Fig. 3B). CrmA, which is the viral caspase 8 inhibitor, similarly inhibited RIP killing and the lethal synergy with c-Myc. Taken together, our data show that FADD and caspase 8 are both required for RIP-induced activation of downstream caspases and for the apoptotic synergy between c-Myc and RIP.

The Apoptotic Activity of RIP Is Conditionally Inhibited by Concomitant Activation of NF-κB—In agreement with previous reports indicating a strong NF-κB-activating function for RIP (22, 29), we found that RIP induced about a 50-fold increase in NF-κB luciferase reporter activity in Rat-1 cells (Fig. 4A). To determine whether activation of NF-κB by RIP influences RIP-
induced cell death, we created Rat-1 cells stably expressing the super-repressor of NF-κB, SRIκBα. This mutant constitutively binds and retains NF-κB in the cytosol, thereby preventing its nuclear translocation and activity as a transcription factor (30). SRIκBα completely abrogated the RIP-induced activation of NF-κB in Rat-1 cells (Fig. 4A). Nonetheless, RIP-induced apoptosis and caspase activation remained unaffected (Fig. 4B). To assess the role of RIP-induced NF-κB more thoroughly, we enhanced the inducibility of NF-κB by stably expressing the p65 NF-κB sub-unit (aka RelA) in Rat-1 cells. In these Rat-1-p65 cells, RIP induced about a 100-fold increase in the NF-κB activity (Fig. 4A). Under such conditions of NF-κB superinduction, RIP induced substantially less apoptosis and DEVD-cleavage (Fig. 4B). The apoptotic sensitivity results did not induce any apoptosis above background, confirming that the observed effect was c-Myc-dependent (data not shown).

Immunofluorescence microscopic examination of both wild type and caspase 8-deficient MEFs with active c-Myc, stained with cyt c-specific antibodies, revealed cells characterized by a diffuse cytosolic pattern of cyt c staining and apoptotic morphology (Fig. 5B, cell on top of the figure shown as an example). In addition, a significant proportion of cells with apparently normal and healthy fibroblast morphology exhibited cytosolic cyt c (Fig. 5B, cell marked with arrow, compare with the neighboring cells exhibiting punctate cyt c staining pattern characteristic of mitochondrial localization). In agreement with our previous data (17), this suggests that c-Myc triggers release of cyt c in the early phase of apoptosis before nuclear collapse or gross apoptotic morphological change. Furthermore, when the mitochondria-containing fractions of MEFs were analyzed by Western blotting using cyt c-specific antibodies, we found that c-Myc activation results in a substantial loss of cyt c from the mitochondrial fractions (Fig. 5C). Together, the data imply that c-Myc promotes cyt c release from mitochondria in a caspase 8-independent manner.

c-Myc and RIP Co-operate to Induce cyt c Release from Mitochondria—Cells with active c-Myc survive in the presence of serum survival factors and, therefore, by inference, c-Myc does not release cyt c in these conditions. It is nonetheless possible that c-Myc still exerts a priming effect on mitochondria that promotes cyt c release upon RIP expression. To determine
whether the RIP-induced apoptotic pathway could exacerbate the mitochondrial effects of c-Myc that induce release of cyt c, we determined the localization of cyt c in Rat-1-MycERTAM cells expressing ectopic RIP.

To ensure widespread RIP expression, cells were subjected to three sequential cycles of infection with freshly harvested RIP-encoding retrovirus. This resulted in 90% efficiency of infection (data not shown). c-Myc was then immediately activated, and the cells were fixed 18 h later. Cells were stained with cyt c-specific antibodies and inspected by immunofluorescence microscopy. A significant number of the cells that expressed both RIP and activated c-MycERTAM exhibited cytosolic cyt c even though their cellular morphology was not apoptotic (Fig. 6A).

We quantitated the proportion of such cells exhibiting “early” cyt c release, which occurs before the onset of apoptosis (cyt c release).

three sequential cycles of infection with freshly harvested RIP-encoding retrovirus. This resulted in >90% efficiency of infection (data not shown). c-Myc was then immediately activated, and the cells were fixed 18 h later. Cells were stained with cyt c-specific antibodies and inspected by immunofluorescence microscopy. A significant number of the cells that expressed both RIP and activated c-MycERTAM exhibited cytosolic cyt c even though their cellular morphology was not apoptotic (Fig. 6A). We quantitated the proportion of such cells exhibiting “early” cyt c release, which occurs before the onset of apoptosis (cyt c

whether the RIP-induced apoptotic pathway could exacerbate the mitochondrial effects of c-Myc that induce release of cyt c, we determined the localization of cyt c in Rat-1-MycERTAM cells expressing ectopic RIP.

To ensure widespread RIP expression, cells were subjected to
Fig. 7. The apoptotic co-operation of oncogenes with RIP requires the mitochondrial pathway of apoptosis. A, lack of Bax and Bak does not inhibit the innate apoptotic activity of RIP but abrogates its apoptotic synergy with c-Myc. pcDNA3.1-RIP was transfected into wild-type MEFs or Bax and Bak double-deficient MEFs followed by activation of c-Myc with OHT. Cell death was assessed as in Fig. 1. B, ectopic expression of Bcl-xL does not inhibit the innate apoptotic activity of RIP but abrogates its apoptotic synergy with c-Myc. pcDNA3.1-RIP was transfected into or without pcDNA3.1-Bcl-xL into Rat-1-MycERTAM cells followed by activation of c-Myc with OHT. Cell death was assessed as in Fig. 1. C, Bcl-xL abrogates the apoptotic synergy between RIP and E2F-1. pcDNA3.1-RIP was transfected into or without pcDNA3.1-Bcl-xL into Rat-1-ER^{ERTAM} cells followed by activation of E2F-1 with various concentrations of OHT. Cell death was assessed as in Fig. 1.

release, no nuclear collapse or gross apoptotic morphological change) and found that these cells were far more frequent in populations expressing both RIP and activated c-Myc than in those expressing either of these pro-apoptotic effectors alone (Fig. 6B). No appreciable numbers of RIP-transduced cells exhibited early cyt c release without c-Myc activation. The early cyt c release upon coexpression of RIP and active c-Myc required caspase activity since it was inhibited by the addition of the caspase inhibitor zVAD-fmk (Fig. 6B).

The Apoptotic Co-operation of Oncogenes with RIP Is Regulated by Mitochondrial BH3 Proteins—To explore the importance of cyt c release for the synergistic apoptosis induction by RIP and c-Myc, we employed MEFs that are refractory to cyt c release due to the lack of both Bax and Bak (31). In wild-type control MEFs activation of MycERTAM induced a 2-fold increase in RIP killing (Fig. 7A). In contrast, although RIP was capable of inducing cell death in Bax/Bak double-deficient MEFs, the ensuing cell death was not augmented by c-Myc (Fig. 7A). We also explored the effects of suppressing cyt c release by expressing Bcl-xL. Our results in Fig. 7B show that although Bcl-xL fails to inhibit the “basal” pro-apoptotic action of RIP in Rat-1 cells (around 30% apoptosis), the apoptotic augmentation conferred by c-Myc activation is completely abolished.

Like c-Myc, E2F-1 also sensitizes cells to death receptor-induced apoptosis (5). To define whether RIP-induced death signals also synergize with E2F-1, ectopic RIP was expressed in Rat-1 cells stably expressing a conditionally active form of E2F-1 (ER^{ERTAM}E2F-1). RIP-induced cell death was enhanced by E2F-1 in a manner depending on the dose of ER^{ERTAM}E2F-1-activating ligand (Fig. 7C). As with c-Myc, Bcl-xL inhibited this apoptotic co-operation between RIP and E2F-1 but not basal RIP killing (Fig. 7C).

Taken together, our data show that c-Myc-dependent augmentation of the apoptotic activity of RIP requires the presence of Bax/Bak and is, therefore, mediated by specific BH3 proteins. By analogy, the same may also apply to E2F-1-dependent augmentation of RIP killing.

DISCUSSION

Activation of c-Myc sensitizes cells to the induction of apoptosis by TNFR, CD95, and TRAIL receptors. One possible mechanism by which c-Myc could induce general death receptor hypersensitivity is by intracellular amplification of a death receptor signal that is otherwise too weak by itself to induce substantial apoptosis. In this case, activation of the death receptor pathway not only by receptor-bound ligand but also by non-receptor-dependent triggers should preferentially kill cells with activated c-Myc.

c-Myc Amplifies the Lethal Effect of Cytosolically Activated Caspase 8 Pathway—To explore the ability of c-Myc to amplify a cytosolically activated death receptor pathway, we ectopically expressed cytosolic components of the death receptor-signaling pathway in cells and asked whether these could synergize with c-Myc to promote apoptosis. We found that apoptosis induction by FADD and pro-caspase 8, which universally link death receptors to the apoptotic machinery (10), and by the TNFR-signaling associated molecule RIP was dramatically enhanced upon c-Myc activation. Apoptotic signaling by TNFR and CD95 is strictly dependent on both FADD and caspase 8 (10). Here we show that RIP-induced apoptosis also strictly requires FADD and caspase 8, implying that ectopic cytosolic expression of RIP kills cells through the same apoptotic pathway as death receptors. Thus, our data suggest that the intersection of synergy between
Apoptotic Synergy between c-Myc and Caspase 8 Pathway

Engaging the Mitochondrial Pathway

High levels of RIP expression and, by inference, lower levels of caspase 8 activation is not blocked by Bcl-2/Bcl-xL. However, the more limited killing triggered by low levels of RIP expression and, by inference, lower levels of caspase 8 activation is greatly augmented if the potential of mitochondria to release cyt c is promoted by c-Myc. There is emerging evidence that in the context of weak caspase 8 activity, cytosolic cyt c (or other pro-apoptotic substance such as Smac/DIABLO released from apoptotic mitochondria) can amplify the ensuing apoptotic response. For example in cell extracts, the presence of mitochondria dramatically lowers the amount of caspase 8 required to activate downstream caspases (33). In addition, a functional mitochondrial pathway is required for caspase-9-induced apoptosis in cells defined as type II cells, in which a low amount of activated caspase 8 is induced upon receptor ligation (32). Moreover, recent evidence has implicated an involvement of Bax and Bcl-2 in the regulation of apoptotic activity of TRAIL, which does not kill primary cells but selectively induces apoptosis in tumor cells (34, 35).

Based on this, we interpret our data as indicating that low levels of RIP expression are insufficient to activate adequate caspase 8 and kill cells directly. However, by promoting cyt c release (independently of caspase 8), activation of c-Myc allows this low level of caspase 8 to engage the mitochondrial pathway, which then amplifies the caspase 8-dependent RIP death signal (Fig. 8). Ligand-mediated activation of TRAIL receptors can activate caspase 8 without concomitant apoptosis (35), which makes our model also applicable to death receptors. Therefore, we propose that c-Myc-induced amplification of FADD and caspase 8-mediated death receptor signals, rather than direct activation of death receptor, is a general mechanism through which c-Myc and death receptors synergize to induce apoptosis (Fig. 8).

The molecular mechanisms through which c-Myc promotes the potential for cyt c release from mitochondria are as yet unclear but are likely to involve c-Myc-dependent changes at the level of expression, localization, or activity of BH3 proteins. Our results show that loss of both Bax and Bak render cells completely refractory to the apoptotic sensitization by c-Myc. Therefore, c-Myc-induced up-regulation of Bax (16) or down-regulation of Bcl-xL or Bcl-2 (36) could “prime” mitochondria to release cyt c. In this case, the most likely candidate linking low level caspase 8 activation to the mitochondrial pathway is the BH3 protein Bid. Bid is cleaved by caspase 8 to its pro-apoptotic functional form tBid, which via oligomerization with the BH3 proteins Bax or Bak then triggers the release of cyt c (31, 37, 38) and engages the downstream Apaf-1/caspase 9 apoptosisome. The role of tBid in the RIP and c-Myc synergy remains to be clarified in further studies.

Cytosolic Death Receptor Pathway Proteins as a Route for the Selective Induction of Apoptosis in Cells Harboring Activated Oncoproteins—Death domains have a propensity to self- and cross-interact via homophilic interactions, and when overexpressed they can auto-oligomerize and induce apoptosis without the need for physiological oligomerization signal like the ligand-induced oligomerization of death receptor (39, 40). Although the exact biochemical mechanism through which RIP triggers the caspase 8 pathway remains to be determined, it is tempting to speculate that the C-terminal death domain of RIP, which was absolutely required for triggering the c-Myc-augmented death pathway (Fig. 1) and that can bind the death domain of FADD (39), mediates oligomerization of ectopically expressed RIP and recruits FADD and caspase 8 in the complex. Therefore, our data showing that both c-Myc and the Rb pathway effector E2F-1 amplify the apoptotic effect of RIP raise the intriguing possibility that pharmacological activation/oligomerization of cytosolic caspase 8 pathway effectors could have a profoundly lethal effect in cells with growth-deregulating mutations while leaving normal cells unscathed.

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