

c-Myc induces cytochrome *c* release in Rat1 fibroblasts by increasing outer mitochondrial membrane permeability in a Bid-dependent manner

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Abstract

Ectopic expression of *c-myc* sensitises cells to a wide range of apoptotic stimuli by inducing the release of cytochrome *c* from the mitochondrial intermembrane space into the cytosol. To elucidate the molecular mechanisms of mitochondrial permeabilisation in response to *c-Myc* activation, we carried out a biochemical fractionation analysis of Rat1 fibroblasts expressing an inducible *c-Myc* protein. We find that cytoplasmic extracts from cells in which *c-Myc* has been activated contain a soluble factor capable of inducing cytochrome *c* release from isolated mouse liver mitochondria. This factor is present only under growth factor deprivation conditions and its activity is inhibited by addition of Bcl-X_L. The *c-Myc*-induced factor copurifies with full-length Bid, a "BH3-only" proapoptotic member of the Bcl-2 family, and antibodies raised against the BH3 domain of Bid inhibit *c-Myc*-induced cytochrome *c* releasing activity. These results are consistent with a model in which the activation of *c-Myc* regulates factors capable of enhancing the mitochondrial membrane destabilisation function of "BH3-only" proteins.

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Keywords: *c-Myc*; apoptosis; mitochondria; cytochrome *c*; Bid; Bax; BH3

Abbreviations: 4-OHT, 4-hydroxytamoxifen; OMM, outer mitochondrial membrane; FCS, foetal calf serum; DMEM, Dulbecco's modified Eagle medium; PMSF, phenylmethylsulphonyl fluoride; IGF-1, insulin-like growth factor 1.

Introduction

The proto-oncogene *c-myc* is involved in a wide range of cellular processes, including proliferation, differentiation and apoptosis. *c-Myc* is a transcription factor belonging to the basic helix–loop–helix zipper (bHLHZ) family. All the activities so far attributed to *c-Myc* require its ability to modulate transcription. In most cases, however, the target genes and the pathways regulated by *c-Myc* remain elusive (see Grandori *et al.*¹ for a review). Among the different *c-Myc* activities, the ability to promote apoptosis is one of the most intriguing. It has been established that rather than inducing apoptosis *per se*, *c-Myc* sensitises many cell types to different apoptotic stimuli.² The ability to promote both proliferation and apoptosis has been proposed as a way to restrain the emergence of neoplastic clones within the soma and seems to be shared by a number of other oncogenes.^{2–4}

The overexpression of the proto-oncogene Bcl-2 strongly cooperates with *c-Myc* both in cell transformation *in vitro* and in oncogenesis *in vivo*.^{5,6} This cooperativity is likely to act by the ability of Bcl-2 to abrogate the apoptotic function of *c-Myc*. Bcl-2 is the founder of a family of proteins with both antiapoptotic (Bcl-2, Bcl-X_L, A1, Mcl1, Bcl-W) and proapoptotic (Bax, Bak, Bid, Bad, Bik, Hrk, Bim, Noxa, Puma) functions.⁷ The antiapoptotic proteins Bcl-2 and Bcl-X_L are integral membrane proteins localised in the mitochondria and the endoplasmic reticulum.^{8,9} Bcl-2, Bcl-X_L and possibly the other antiapoptotic members of the family protect mitochondrial integrity by regulating the outer mitochondrial membrane (OMM) permeability and the activity of metabolite exchange channels.^{10,11} The mechanism by which Bcl-2 and Bcl-X_L exert their protective role is currently unknown, but it seems to require domains affecting the heterodimerisation with proapoptotic members of the same family (Bax, Bak, Bid, etc.) as well as domains affecting a putative channel-forming activity.¹²

The proapoptotic members of the Bcl-2 family fall into two subsets. The so-called multidomain factors are proteins sharing more than one homology domain, like Bax and Bak. The other subfamily comprises proteins sharing only the BH3 domain. These 'BH3-only' proteins are often downstream effectors of signalling pathways. They are usually constitutive active peptides inactivated by survival signals or freed from inactivators following diverse cellular stresses. For example, Bad is sequestered in an inactive cytoplasmic complex by 14-3-3 after being phosphorylated by survival kinases.¹³ Bim is kept separate from mitochondria by binding to dynein light chain in healthy cells and is released after cellular stress, migrating to mitochondrial membranes.¹⁴ In the multidomain subfamily Bak is an integral OMM protein both under normal and apoptotic conditions. Bax, early after the apoptotic

stimulus, migrates to the OMM following a conformational change. The 'BH3-only' protein Bid may be involved in mediating the conformational change of Bax.¹⁵ Destabilisation of the OMM following Bax conformational change results in the release of cytochrome *c* and other apoptotic proteins from the intermembrane space, as well as activation of the caspase-9-dependent apoptotic pathway.

Cytochrome *c* release from the mitochondria is a key step in c-Myc-induced sensitisation to apoptosis.¹⁶ Presumably, c-Myc activation has a direct effect on the OMM permeability, reducing the threshold for a complete membrane destabilisation and therefore cooperates with other stimuli that trigger the mitochondrial apoptotic pathway.

The mechanism by which c-Myc destabilises the OMM is not known. It might involve the upregulation of cytosolic factors causing membrane destabilisation or the downregulation of protective factors located in the mitochondria. In the present study, we approach the problem by using an *in vitro* fractionation analysis to discern between these two possibilities. We show that cytosolic extracts from Rat1 fibroblasts expressing the conditional c-Myc fusion protein (MycERTM) contain a soluble factor capable of releasing cytochrome *c* from isolated mouse liver mitochondria. This is present only under conditions where c-Myc has been activated. Fractionation studies show that this factor copurifies with full-length Bid, a proapoptotic member of the Bcl-2 family. We propose that c-Myc regulates factors capable of enhancing the membrane destabilisation function of 'BH3-only' proteins.

Results

Using a chimeric protein obtained by fusing c-Myc with a modified version of the oestrogen receptor (MycERTM),¹⁷ it has been shown that activation of c-Myc is followed by an early redistribution of cytochrome *c* from the mitochondria to the cytoplasm.¹⁶ The mechanisms by which c-Myc increases mitochondrial membrane permeability could involve regulation of cytosolic factors or a direct action on a component of the mitochondrial membrane. To discriminate between these two possibilities, we made use of mitochondria purified from a source external to the system. We set up an *in vitro* assay in which mitochondria purified from mouse liver are incubated with the soluble cytoplasmic fraction of cell extracts from Rat1 fibroblasts expressing MycERTM. We reasoned that if a cytosolic factor responsible for cytochrome *c* relocalisation is a c-Myc-regulated target, this should induce a similar relocalisation in mitochondria purified from mouse liver. c-Myc-induced apoptosis in Rat1/MycERTM cells starts to be evident after 8 h of 4-hydroxytamoxifen (4-OHT) addition (Figure 1), and at this time, cells with relocalised cytochrome *c* start to appear in the cell population.¹⁶ We therefore decided to analyse extracts from cells incubated for 8 h with 4-OHT. As shown in Figure 2a, after 1-h incubation almost 90% of the cytochrome *c* was released from the mitochondria incubated with these extracts (+4-OHT lanes). Interestingly, the cytochrome *c* releasing activity could be almost totally inhibited by the addition of recombinant Bcl-X_L protein. Rat1/MycERTM cells grown in the presence of 10% serum do not show any apoptotic features, even in presence of 4-OHT (data not

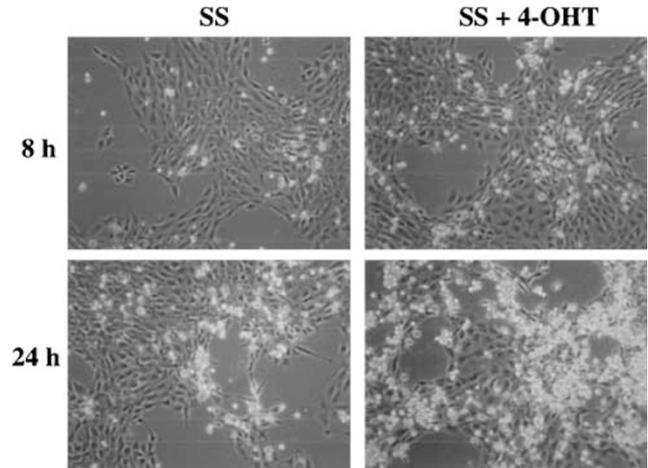


Figure 1 Apoptosis induced by activation of c-Myc in serum-starved Rat1 fibroblasts. The Rat1/MycERTM clone G2¹⁷ was serum starved for 24 h. In the right panels, 100 nM 4-OHT was added to the samples in serum-free medium, respectively, for 8 and 24 h (SS+4-OHT). Pictures of serum starved-only cells (SS) are shown in the left panels

shown). Protein extracts from cells grown in 10% foetal calf serum (FCS) (Figure 2, lane 10% FBS + 4-OHT) are essentially devoid of cytochrome *c* releasing activity.

Serum-starved Rat1/MycERTM cells grown in the absence of 4-OHT showed some degree of cytochrome *c* releasing activity (data not shown). This could be because of either lack of specificity of the activity found or leaky regulation of the inducible system used (ER fusion). To discriminate between these two possibilities, we measured the cytochrome *c* releasing activity of extracts obtained from cells serum starved for 24 h and then treated or untreated for 8 h with 4-OHT. The results shown in Figure 2b demonstrate that addition of 4-OHT to the cells is responsible for a three-fold increase in activity, suggesting a direct connection between c-Myc activation and cytochrome *c* releasing activity. Furthermore, extracts from Rat1 fibroblasts containing only the vector plasmid (pBABEpuro) do not show any cytochrome *c* releasing activity, even after 48 h of serum starvation, suggesting that the observed activity is c-Myc-specific (Figure 2a, Rat1 SS).

Based upon the above experiments, we started a fractionation of cytoplasmic extracts from Rat1/MycERTM cells that had been serum starved 24 h and treated with 4-OHT for 8 h. When the cell extract was loaded onto a Q Sepharose column, a cytochrome *c* releasing activity eluted as a single peak at around 300 mM NaCl (Figure 3, panel A). The pooled active fractions were further fractionated using a Superdex 200 gel filtration chromatography column. The activity eluted as a single peak with an elution volume corresponding to an apparent molecular weight (MW) of 25 kDa (Figure 3, panel B).

Several proapoptotic members of the Bcl-2 protein family have been shown to induce the release of cytochrome *c* from purified mitochondria *in vitro*.^{19–21} In order to determine if one of these proteins is responsible for the c-Myc-dependent cytochrome *c* releasing activity in Rat1 fibroblasts, we probed active fractions from both columns for the presence of the

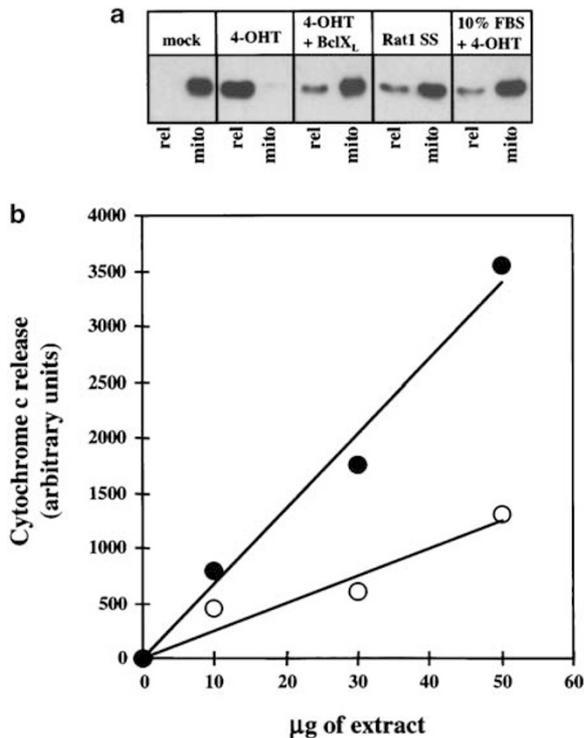


Figure 2 Extracts of cells expressing c-Myc induce cytochrome *c* release from purified mouse liver mitochondria. (a) Analysis by immunoblot for cytochrome *c*. Purified mitochondria were incubated with cytosolic cell extracts as described in Materials and Methods. After centrifugation, both the supernatant (rel) and the pellet (mito) were analysed. The 'mock' sample was incubated with reaction buffer only. The following extracts were used: Rat1/MycERTM cells serum starved for 24 h and incubated with 4-OHT for 8 h (4-OHT); the same extract in the presence of 4 ng of recombinant Bcl-X_L (4-OHT+Bcl-X_L); Rat1/pBABE serum starved for 24+8 h (Rat1 SS); Rat1/MycERTM cells grown in 10% FBS and treated with 4-OHT for 8 h (10% FBS+4-OHT). (b) Increasing amounts of protein extracts were tested for their ability to induce the release of cytochrome *c* from purified mitochondria at 0, 1, 2 and 3 h of incubation. The rate of the release (amount of cytochrome *c* released per hour) was plotted against the amount of extract used. Extracts from Rat1MycERTM cells treated with 4-OHT are indicated by filled circles. Extracts from the same cells, serum starved only, are indicated by open circles

soluble members of the Bcl-2 protein family, Bax and Bid. As shown in Figure 4, the cytochrome *c* releasing activity coeluted with Bid, both from the Q Sepharose column and from the S200 column.

Bid is a 23-kDa protein that has been shown to be cleaved following caspase-8 activation. Bid cleavage induces a more than 10-fold increase in cytochrome *c* releasing activity with respect to full-length Bid, both *in vitro* and *in vivo*.^{19,22} It is therefore possible that c-Myc regulates cytochrome *c* release in the cells by modulation of the cleavage status of Bid. Alternatively, c-Myc may, directly or indirectly, induce an increase in Bid expression level. As shown in Figure 5 (panel a), no trace of the 15 kDa Bid cleavage product is present in Rat1/MycERTM extracts after addition of 4-OHT. The only immunoreactive material detected runs with an apparent MW of 23 kDa, corresponding to the full-length Bid. The same apparent MW is associated with the Bid immunoreactive band present in the partial purified activity (Figure 5, panel a, lane pool QFF). To show that, despite the absence of Bid cleavage product after 4-OHT addition, rat Bid could be cleaved by

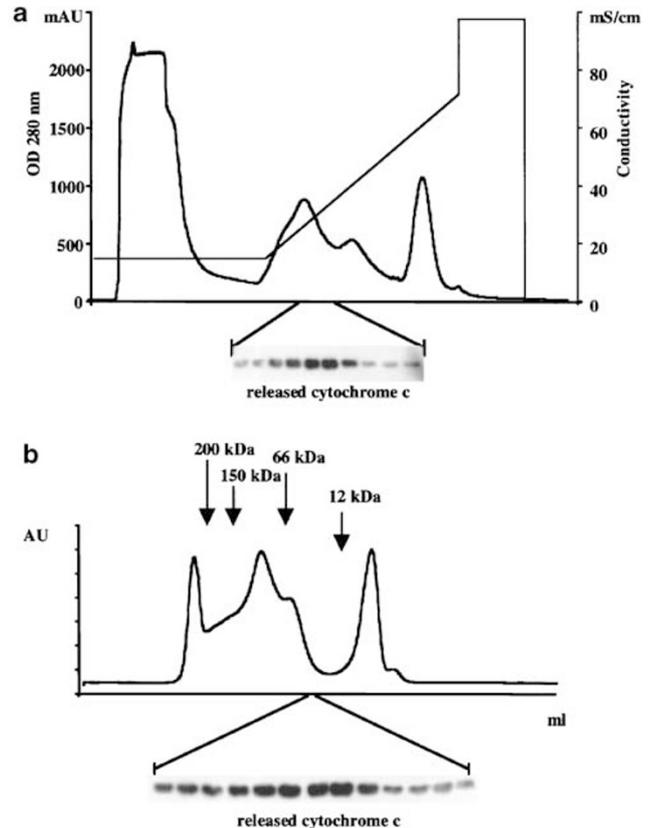


Figure 3 Chromatographic analysis of the c-Myc-induced cytochrome *c* releasing activity. In panel a, 200 mg of protein extract from 4-OHT-treated Rat1MycERTM cells were loaded onto a 20 ml QFF anion exchange column. After washing, the column was eluted with a linear salt gradient. An aliquot of the fractions was tested for cytochrome *c* releasing activity on purified mitochondria. The fractions active for cytochrome *c* release eluted around 300 mM NaCl. Panel b, active fractions from the QFF column were pooled, concentrated and loaded on a 26/30 S200 Gel filtration column. The cytochrome *c* releasing activity eluted with an apparent native MW of 25 kDa. The column had been previously calibrated using the following proteins as MW standards: β -amylase, alcohol dehydrogenase, bovine serum albumin, carbonic anhydrase and cytochrome *c*

caspase-8, we incubated the same extract with recombinant active caspase-8. As shown in Figure 5 (panel b), after addition of recombinant caspase-8, the 23-kDa Bid immunoreactive band is completely converted to a faster migrating band with an apparent MW of 15 kDa. Figure 5 also shows that the induction of c-Myc does not produce any increase of Bid protein level, excluding the possibility that Bid is transcriptionally regulated by c-Myc.

Proapoptotic members of the Bcl-2 protein family have been shown to be regulated at the post-translational level. Bad, for example, is inactivated by phosphorylation through the action of Akt and several other kinases.^{13,23} Bid itself has been shown to be myristoylated after cleavage,²⁴ and recent evidence for phosphorylation has also been reported for Bid.²⁵ c-Myc activation might therefore regulate Bid activity by changing its post-translation modification status. To answer this question, we performed a two-dimensional electrophoresis analysis on cytoplasmic extracts from Rat1/MycERTM cells treated with or without 4-OHT for 8 h in the absence of serum. On a 3–10 pH range the only Bid immunoreactive

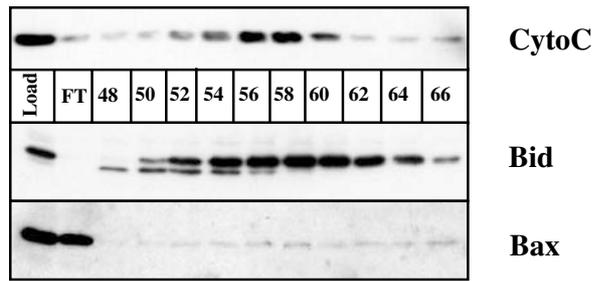
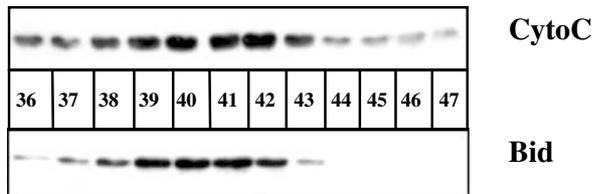
a Anion exchange chromatography**b Size exclusion chromatography**

Figure 4 The proapoptotic member of the Bcl-2 protein family, Bid, copurifies with the cytochrome *c* releasing activity. Fractions from the QFF column (panel a) and from the S200 column (panel b) are tested for the presence of the proapoptotic proteins Bid and Bax. In the upper part of each panel is shown the amount of cytochrome *c* released from purified mouse mitochondria after incubation with the indicated samples

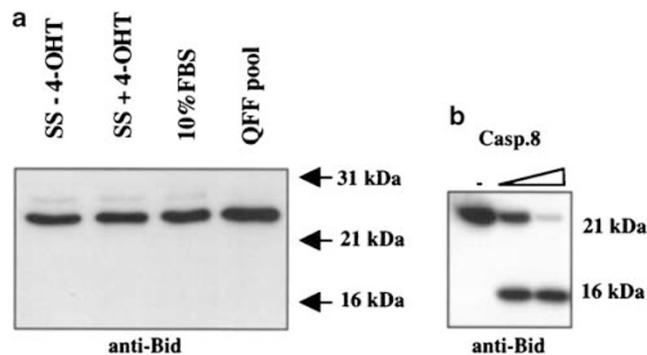


Figure 5 Induction of c-Myc does not increase Bid level or induce its cleavage. Immunoblot analysis of endogenous Bid in Rat1 cell extracts. In lanes 1 and 2 (panel a), the cells were serum starved for 24 h and then incubated for 8 h in the presence or absence of 4-OHT, in serum-free medium. In lane 3, the cells were grown in the presence of 10% FBS. Lane 4 was loaded with an aliquot of the pool from the QFF column used to fractionate the protein extract shown in lane 2, and containing all the cytochrome *c* releasing activity. In panel b, 30 μ g of extract were treated with 20 and 100 ng of recombinant active caspase-8 (Pharmingen) for 30 min at 30°C

material was a single spot with a *pI* around 5. When we narrowed the pH range to a single unit, Bid was focusing as a doublet, with a pattern typical of a phosphorylation shift (Figure 6). Bid has recently been shown to be phosphorylated by casein kinase I and II.²⁵ The phosphorylation has been shown to be on the Ser61 and Ser64 and to be responsible for an increased resistance of the protein to caspase-8 cleavage. Figure 6 shows that, despite the presence of a phosphorylation pattern associated with rat Bid, no difference was

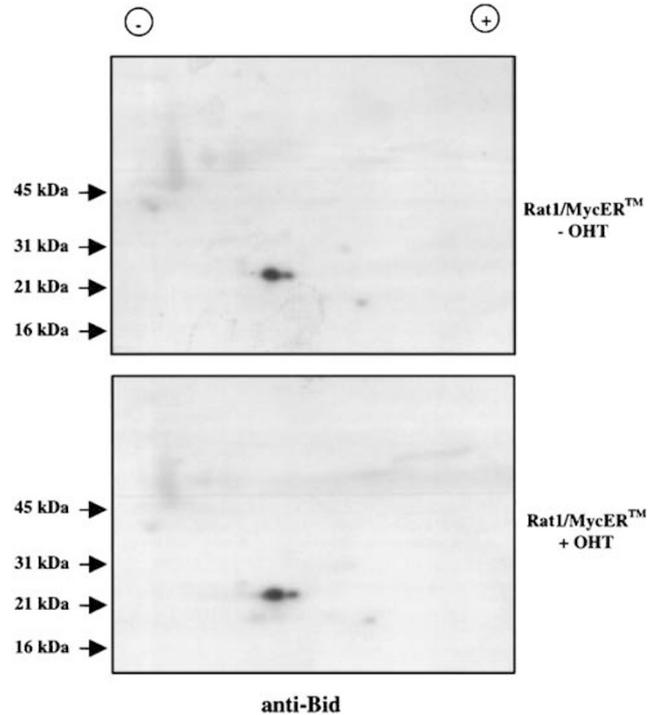


Figure 6 2D-gel analysis of Rat1/MycERTM cell extracts. Immunoblot analysis of endogenous Bid in Rat1 cell extracts. Total extracts from 2×10^6 cells were focused on a pH 3–10 18 cm IEF strip (Amersham Biosciences). A 7 cm length of the strip corresponding to the pH interval 5–6 were cut and loaded on a 15% SDS-PAGE. Extracts from Rat1/MycERTM cells serum starved 24 h and then treated (lower panel) or not (upper panel) with 100 nM 4-OHT were analysed.

detectable between extracts of Rat1/MycERTM cells treated or not with 4-OHT.

Since c-Myc does not induce changes in the level of expression of Bid or its post-translation modification, we investigated the possibility that Bid could be bound in an inactive complex under conditions in which c-Myc is not active, and released upon induction of c-Myc. We performed a gel filtration analysis on extracts from Rat1/MycERTM treated with or without 4-OHT reasoning that, if Bid is complexed with an 'inactivator', this should result in a shift in apparent MW on the gel filtration analysis. No difference in the elution volumes profiles was detectable using size exclusion chromatography (data not shown), suggesting that if such an inactivator exists it must be relatively small.

To address the possibility that c-Myc could regulate the expression or the activity of a factor essential for Bid functioning, we produced recombinant Bid and used it in complementation studies. As has been shown previously,²⁶ purified recombinant Bid is incapable of inducing cytochrome *c* release from mouse liver mitochondria in the absence of Mg ions (Figure 7a, lane 1). To investigate if cell extracts could complement Bid for cytochrome *c* releasing activity, we added to the same amount of purified Bid protein different cytoplasmic extracts. In this experiment, extracts from Rat1 fibroblasts grown in the presence of serum (lane 2) or extracts from Rat1/MycERTM cells grown in the absence of 4-OHT (lane 3) show no cytochrome *c* releasing activity. To detect a possible increase in cytochrome *c* releasing activity because

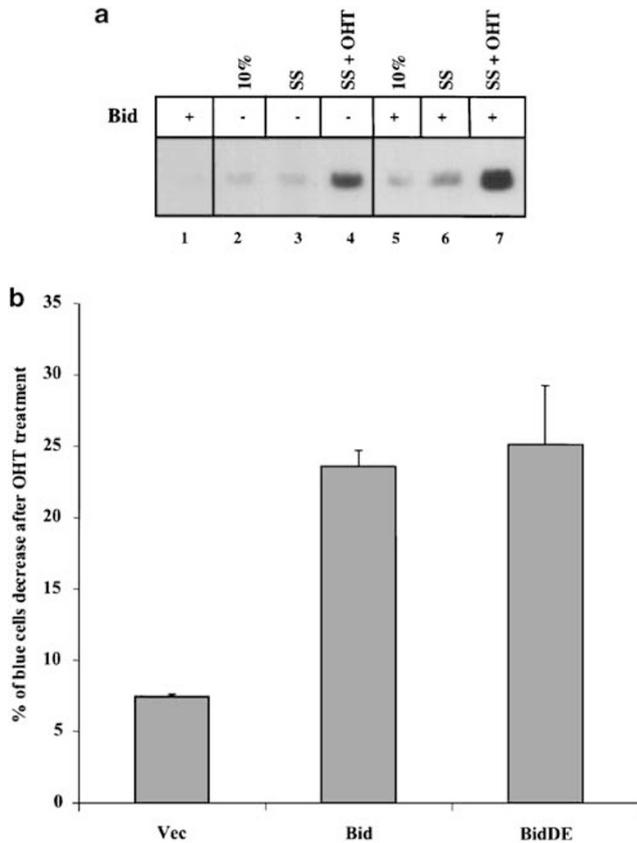


Figure 7 Full-length Bid synergises with c-Myc both *in vitro* and *in vivo*. Immunoblot analysis of cytochrome *c* released from purified mouse liver mitochondria (panel a). Mitochondria were incubated either with 3 pmoles of recombinant Bid alone, or Bid together with 30 μ g of the indicated cell extracts. Lanes 2 and 5: extract from Rat1/MycERTM cells grown in 10% serum; lanes 3 and 6: extract from Rat1/MycERTM cells serum starved for 24 h; lanes 4 and 7: extract from Rat1/MycERTM cells serum starved for 24 h and treated with 4-OHT for 8 h. In panel b, Rat1/MycERTM cells were cotransfected with the LacZ reporter gene and either the empty vector (Vec), or the vector expressing wt Bid (Bid) or a noncleavable mutant of Bid (BidDE). Cells were starved in 1% FCS for 24 h and then treated with or without 4-OHT for 16 h in 1% FCS. The percentage of blue cells was counted in each sample. The graph, result of the experiment carried in duplicate, shows the percentage decrease in blue cells over the total in samples treated with 4-OHT with respect to the untreated controls. A 4-OHT-specific reduction in the number of blue cells over the total is a measure of synergism between c-Myc and the tested proteins

of the presence of recombinant Bid in the reaction, conditions were chosen to give nonsaturated cytochrome *c* release. Using this approach, extracts from Rat1/MycERTM cells grown in the presence of 4-OHT induce the release of only 30–40% of the total cytochrome *c* from mitochondria (lane 4 and data not shown). As shown in Figure 7a (lane 7), only extracts in which c-Myc has been activated produced a significant increase in Bid activity. The very small activation given by the extract from Rat1/MycERTM cells grown in the absence of OHT (compare lane 3 and 6) could be explained by the slight leakiness of the system (compare with Figure 2b).

The synergy of extracts containing activated c-Myc with pure recombinant Bid in eliciting cytochrome *c* releasing activity and the absence of any Bid cleavage product in this system (see Figure 5) led us to test if Bid and a noncleavable Bid mutant (Bid D60E) was able to exacerbate c-Myc-induced

apoptosis. In order to do this, pcDNA3.1, pcDNA3.1/BIDhis or pcDNA3.1/BID-D60Ehis were cotransfected together with pcDNA3.1/LacZ, as a gene reporter, into Rat1/MycERTM cells. After serum starvation and 4-OHT treatment, cells were fixed and stained for β -galactosidase activity. As shown in Figure 7b, the percentage of blue-stained cells, upon Bid cotransfection (Bid), showed a significant and reproducible decrease after 4-OHT treatment, compared with the sample cotransfected with vector alone, indicating that c-Myc-induced sensitisation to apoptosis was further increased by the expression of Bid. The same decrease was observed in cells cotransfected with a plasmid carrying Bid mutated in the caspase-8 cleavage site (Bid D60E), indicating that the synergistic effect observed in this experiment is independent of caspase-8-mediated Bid cleavage.

The data presented in Figure 7 suggest that activation of c-Myc does not influence Bid directly, but that it regulates a second soluble factor that, in turn, regulates Bid activity. In this scenario, Bid could be dispensable for c-Myc-induced cytochrome *c* activity, if another proapoptotic member of the Bcl-2 protein family could substitute for it. To address this question, we used antibodies directed against the BH3 domain of Bid to inhibit c-Myc-induced cytochrome *c* activity. As shown in Figure 8, an antibody recognising the Bid BH3 domain inhibits the c-Myc-induced cytochrome *c* releasing activity in a dose-dependent manner. The same result was obtained using a commercial antibody raised against full-length Bid peptide (data not shown). In contrast, no inhibitory effect was seen using the same amount of an antibody directed against the BH3 domain of the proapoptotic Bcl-2 family protein Mtd/Bok, suggesting that Bid is necessary for cytochrome *c* release in this system.

We have shown that Bid plays an important role in the cytochrome *c* relocalisation induced by c-Myc. It was recently shown that in HeLa cells, Bid induces the oligomerisation and insertion of Bax in the OMM by changing its conformational status.¹⁵ It is possible to detect conformational changes of Bax *in vivo* using a monoclonal antibody raised against its N-terminus (Mab 6A7).^{15,27,28} The epitope recognised by Mab 6A7 becomes exposed only after an apoptotic stimulus. We therefore investigated whether activation of c-Myc could influence the conformational status of Bax *in vivo*, by measuring the immunoreactivity of Bax to the Mab 6A7 in Rat1/MycERTM cells treated with or without 4-OHT. As shown in Figure 9, activation of c-Myc markedly increases the number of cells positive for Mab 6A7 staining, suggesting that c-Myc promotes conformational activation of Bax.

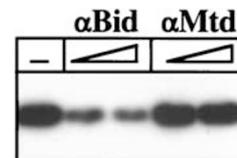


Figure 8 Antibodies raised against Bid BH3 domain inhibit c-Myc-induced cytochrome *c* releasing activity. Immunoblot analysis of cytochrome *c* released from purified mouse liver mitochondria. Mitochondria were incubated with extract from Rat1/MycERTM cells serum starved for 24 h and treated with 4-OHT for 8 h, in the presence of increasing amounts (2 and 5 μ g) of purified polyclonal antibodies raised against the BH3 peptides of Bid (aa 82–106 of the mouse sequence) or MTD/Bok (aa 63–87 of the mouse sequence)

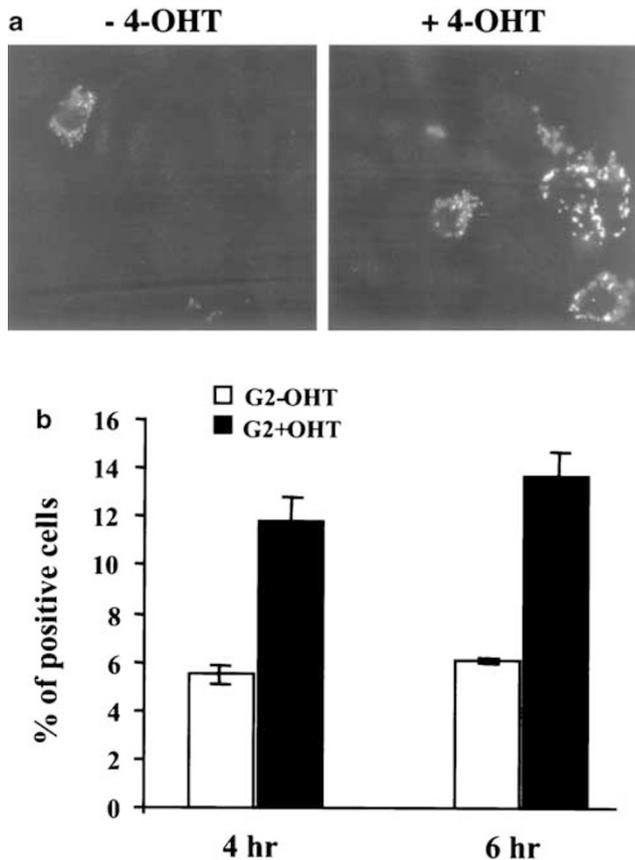


Figure 9 Treatment of Rat1/MycERTM with 4-OHT induces a Bax conformational change. Immunofluorescence analysis of endogenous rat Bax in Rat1/MycERTM cells treated or untreated with 4-OHT for 4 and 6 h. Cell monolayers were fixed by addition of 1% paraformaldehyde in PBS. Coverslips were washed in PBS and incubated with 6A7 anti-Bax monoclonal antibody (Trevigen). Panel a: representative pictures of cells treated (+4-OHT) or untreated (-4-OHT) with 4-OHT for 6 h. The appearance of a strong perinuclear punctate staining is a measure of a conformational change of Bax. Panel b: percentage of Bax-positive cells quantified by counting a minimum of 500 cells in five randomly chosen fields. Data from untreated cells (G2-OHT) and 4 h- or 6 h-4-OHT treated cells (G2+OHT) is displayed. Positive cells are expressed as a percentage of the total number of cells analysed. Values represent the mean and variation of a typical experiment performed in duplicate.

Discussion

In order for a cell to become transformed, it must acquire new proliferative capabilities and concomitantly be able to escape apoptotic cell death. Genetic lesions that increase the proliferative capability of cells often affect also their sensitivity to apoptotic stimuli.²⁹ This is particularly well described for the oncogene *c-myc*. Ectopic expression of *c-myc* seems to have a dual effect: on the one hand, it is sufficient to drive cells into mitosis, while on the other hand, it sensitises cells to many apoptotic stimuli, such as growth factor deprivation, Fas ligation, UV damage, TNF α and hypoxia. A better understanding of the molecular pathways downstream of c-Myc could, therefore, give an invaluable insight into the comprehension of the process of neoplastic transformation.

A step towards understanding the way c-Myc sensitises cells to apoptosis came from previous studies.¹⁶ Using an inducible MycER fusion protein, it was shown that c-Myc

triggers the release of cytochrome *c* from the intermembrane mitochondrial space into the cytosol. This process is caspase independent and blocked by the survival factor (IGF-1) insulin-like growth factor 1. Interestingly, c-Myc-induced apoptosis could be blocked by microinjection of anti-cytochrome *c* antibodies, and microinjection of holocytochrome *c* in the cytoplasm of the cells mimicked the effect of c-Myc activation, thus sensitising cells to other apoptotic stimuli.¹⁶

The mechanism of c-Myc-induced release of cytochrome *c* from mitochondria may involve either the positive regulation of factors with membrane destabilising action or downregulation of proteins that protect mitochondrial homeostasis, or both. The sensitisation effect shown by c-Myc expression under conditions of growth factors deprivation could be the result of a complex signalling network that affects both mitochondrial and cytosolic factors. We analyse in this paper the effect of cytosolic extracts, obtained from cells in which c-Myc is ectopically expressed, on mitochondria purified from a source external to the system. We observe that cytoplasmic extracts are capable of inducing the release of cytochrome *c* from the mitochondrial intermembrane space only when they are derived from cells in which c-Myc has been previously activated by the drug 4-OHT. These findings demonstrate that c-Myc modulates soluble cytosolic factors with cytochrome *c* releasing activity. The possibility that c-Myc acts also on mitochondrial components has not been addressed directly.

IGF-1 is a potent inhibitor of c-Myc sensitisation to growth factors deprivation-induced apoptosis.³ It has been observed that IGF-1 inhibits the release of cytochrome *c* from mitochondria induced by c-Myc activation. IGF-1 is a survival factor that activates both the MAPK pathway and the PI3K/Akt pathway through the binding to its receptor tyrosine kinase. Both pathways have been proposed to protect cells from several apoptotic stimuli.³⁰ Suppression of c-Myc-induced apoptosis in Rat1 fibroblasts seems to depend exclusively on the PI3K/Akt pathway.³¹ Activation of Akt leads to the engagement of multiple antiapoptotic pathways, either transcription dependent or transcription independent.³² Akt has been shown recently to play a pivotal role in the control of mitochondrial homeostasis by regulating metabolite exchange through the outer membrane.^{33,34} If IGF-1 exerted its protective role solely on the mitochondria and not only on cytosolic constituents, then, in our *in vitro* system we would expect no difference in the cytochrome *c* releasing activity of extracts from cells grown in the presence of 4-OHT with or without serum in the medium. In contrast, we observe an almost total absence of cytochrome *c* releasing activity when serum was added to the medium, suggesting a more complex action of serum and survival factors in general. It is interesting to speculate that survival factors might act by influencing the pattern of gene expression induced by c-Myc. In line with this hypothesis is the observation that IGF-1 was found to suppress phosphorylation of threonine 58 in the c-Myc transactivation domain. Interestingly, a T58A mutant showed a drastically reduced proapoptotic activity and an increased transforming activity.³⁵

We found that the proapoptotic member of the Bcl-2 family, Bid, coeluted, at least in two chromatographic steps, with the c-Myc-induced cytochrome *c* releasing activity. In contrast,

Bax was present in the flow through of the Q Sepharose column and it was almost undetectable in the active fractions (Figure 4). We also found that Rat1/MycER™ cells grown in the presence of 4-OHT showed increased immunoreactivity to an antibody that detects a Bax change in conformation. Bax conformational change has been related to its translocation to the mitochondria and induction of cytochrome *c* release. Therefore, although Bax is dispensable for cytochrome *c* release *in vitro*, we find that it participates in the destabilisation of mitochondria in the intact cells. This apparently contradictory result may be explained by the redundancy in function of the 'multidomain' proapoptotic members of the Bcl-2 family, Bak and Bax. Although these proteins show different subcellular localisation in healthy cells, they multimerise and insert into the OMM following a 'BH3 only' protein-induced conformational change.^{36–38} It is therefore possible that Bak, which is constitutively present in the purified mouse mitochondria as an integral membrane protein, may replace the Bax protein present in the rat fibroblast extracts in our *in vitro* assay.

The fact that Bid coeluted with the c-Myc-induced cytochrome *c* releasing activity initially appeared to tie in with data showing that Fas receptor ligation was essential for c-Myc-induced death.³⁹ This work implicated receptor-mediated activation of caspase-8, cleavage of Bid and induction of cytochrome *c* release with consequent apoptosome formation and caspase-9 activation in c-Myc-induced apoptosis. In the present study, however, two main observations support a role in apoptosis for full-length Bid in Rat1 fibroblasts: (a) we were unable to detect any truncated Bid after c-Myc activation following 4-OHT treatment and (b) using LacZ cotransfection experiments, we observed synergy in the induction of apoptosis between a cleavage-resistant Bid mutant and c-Myc activation (Figure 7b). Rat1 fibroblasts are, in general, refractory to Bid cleavage in response to apoptotic treatments, although Bid from these cells could be cleaved *in vitro* using purified caspase-8 (Figure 6b and data not shown). The lack of Bid cleavage in cells following c-Myc activation, nevertheless, fits with the observation that cytochrome *c* release induced by c-Myc is caspase independent¹⁶ supporting a model in which Fas ligation cooperates with c-Myc on a parallel pathway.^{16,29} The activity of full-length Bid in this system can therefore only be explained either by invoking a second, as yet unknown, way for Bid to be activated, or by the presence of a coactivator in extracts of cells in which c-Myc has been activated.

It has been shown that caspase-8-induced cleavage products of Bid remain bound in a noncovalent complex. The NH₂-terminal glycine generated by the cleavage becomes a substrate for myristoylation, which in turn targets the protein to mitochondria.²⁴ Although no major change in Bid conformation has been observed *in vitro* after caspase-8 cleavage,⁴⁰ we are not able to exclude the possibility that an increased flexibility of Bid structure, induced by the cleavage, could help the BH3 domain to become a better ligand for the multidomain Bcl-2 family members, Bax and Bak. In this scenario, one could imagine the existence of a small ligand that would produce a similar change in Bid conformation without affecting its cleavage status. It would be interesting to investigate whether small lipids such as cardiolipin or

phosphatidylglycerol, both of which have been shown to bind Bid,^{41,42} could induce such a conformational change. Alternatively, the binding of such lipids could increase the ability of full-length Bid to target mitochondrial membranes. In line with this notion we have observed, by immunofluorescence, an apparent increase in mitochondrial localisation of endogenous Bid following c-Myc activation (data not shown). Non-covalent interactions of Bid with small molecules of this type may not result in altered migration on sizing columns or 2D gels.

We considered a further model to explain c-Myc modulation of Bid activity. In this model, c-Myc would modulate the expression or activity of another factor essential for Bid to induce cytochrome *c* release. In support of this possibility, we show that extracts in which c-Myc has been switched on are capable of 'activating' recombinant Bid added to the reaction. This apparent activation may also depend on the presence in the extracts of molecules responsible for a possible completion of cytochrome *c* release triggered by Bid. In support of this hypothesis, Ott *et al.*⁴³ have recently shown that cytochrome *c* release from mitochondria proceeds by a two-step process. Alternatively, c-Myc may regulate a soluble regulator of the putative cytochrome *c* permeability channel. Cyclophilin D, a component of the permeability transition pore (PTP), implicated in many forms of cell death,⁴⁴ has been shown to be upregulated by c-Myc in microarray studies.⁴⁵ Using a similar approach, our own studies show upregulation of cyclophilin D in human cells in which c-Myc has been activated. We also found that VDAC and the benzodiazepine receptor, two other components of the PTP, are upregulated by c-Myc (II and JD, unpublished observations). The possibility that c-Myc regulates a component of the cytochrome *c* releasing channel could significantly change our model of c-Myc-induced cytochrome *c* release, implying direct c-Myc effects on the mitochondria as well as cytosolic constituents.

In a linear model in which c-Myc activates Bid, which in turn induces mitochondrial destabilization, Bid could be indispensable for c-Myc-induced apoptosis. Although we were not able to address this issue directly in Rat1 fibroblasts, it is possible that in intact cells or whole animals, other BH3-only proteins could contribute significantly to this process, even though Bid is clearly the most important one in our *in vitro* system. Bid may not, therefore, be universally required for c-Myc-induced cell death, but may contribute to differing extents in different cell types. c-Myc could modulate the activity of factors essential for the functioning of a subset of BH3 proteins, or the whole BH3-only family in general.

Materials and Methods

Cell culture

Rat1 fibroblasts expressing the 4-OHT conditional allele of *c-myc* (Rat1/MycER™) have been described previously.¹⁷ Cells were grown in Durbecco's modified Eagle medium (DMEM) supplemented with 10% FCS. Following serum deprivation for 24 h, c-Myc was activated by addition of 100 nM 4-OHT (Sigma) in serum-free medium. For protein purification, cells were grown in 30 × 1800 cm² roller bottles, containing 200 ml medium. Bottles were previously equilibrated in 5% CO₂.

Subcellular fractionation

Cells from roller bottles were washed with phosphate buffer saline (PBS) and trypsinised for 5 min at 37°C. Trypsin was neutralised with DMEM supplemented with 30% FCS. After centrifugation, cells were washed once with cold PBS supplemented with 1 mM Phenyl-methyl sulphonyl fluoride (PMSF) (Sigma), and briefly washed in lysis buffer (20 mM HEPES/KOH pH 7.4, 100 mM sucrose, 0.1 mM EGTA, 5 mM MgCl₂, 1 mM DTT, 1 mM PMSF and 1 × Complete™ [Roche]). Cells were incubated for 30 min on ice at a concentration of 40 × 10⁶ cells per ml of lysis buffer, and then Dounce homogenised until 80–90% of the cells were trypan blue positive. Nuclei and unbroken cells were centrifuged for 10 min at 1500 × *g*. The supernatant was further centrifuged for 30 min at 10,000 × *g* to pellet the heavy membrane fraction. The soluble fraction was passed through a 0.22 μm filter and processed for Western blot analysis or protein purification.

Purification of mitochondria from mouse liver

Mitochondria were purified essentially as described by Hovious *et al.*¹⁸. Briefly, two livers were homogenised in 300 mM sucrose, 10 mM HEPES buffer pH 7.4. Nuclei and debris were spun down at 600 × *g* for 5 min. Mitochondria and heavy membranes were pelleted by centrifugation at 8700 × *g* for 10 min. The pellet was washed once in the same buffer supplemented with 1 mM PMSF and resuspended in 2 ml of mannitol–MOPS buffer (300 mM mannitol, 5 mM MOPS pH 7.2). The suspension was banded in a 70/30/10% Percoll (Sigma) step gradient in mannitol–MOPS buffer by centrifugation for 30 min at 14 400 × *g* (Beckmann SV40 Ti rotor, brake position zero). Preparation purity was verified by electron microscopy.

Cytochrome *c* release assay

The ability of cytoplasmic extracts to induce release of cytochrome *c* from purified mitochondria was assessed by incubating 30 μg of mitochondria with 30 μg of extracts from cells treated with or without 4-OHT in 30 μl final volume. The reaction was carried at 35°C for 1 h in reaction buffer (250 mM mannitol, 100 mM sucrose, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 20 mM HEPES/KOH pH 7.4) and then centrifuged at 9000 × *g* for 5 min. The mitochondrial pellet was resuspended in 30 μl of reaction buffer containing 1% Triton. For the measurement of the cytochrome *c* rate of release, 10, 30 and 50 μg of extract from Rat1/MycER™ cells treated with or without 4-OHT were incubated for 1, 2 and 3 h with 30 μg of pure mitochondria. The amount of cytochrome *c* released was quantified by densitometry. The initial velocity was calculated graphically and plotted against the amount of extract used.

Western blot analysis

To analyse the amount of cytochrome *c* released from purified mouse liver mitochondria, 2 μl of reaction supernatant or solubilised pellet were loaded on a 15% SDS-PAGE. Gels were transferred on Immobilon PVDF membranes (Millipore) and stained for cytochrome *c* using the monoclonal antibody 7H8.2C12 (Pharmingen). The presence of Bid in the chromatographic fractions was tested by using a goat polyclonal anti-Bid antibody (R&D Systems). Bax antibody (TL41) was produced by immunising rabbits with a KLH-conjugated peptide spanning amino acids 57–72 around Bax BH3 domain.

Protein purification

Cytoplasmic extracts from 3 × 10⁹ cells were brought to a conductivity corresponding to 150 mM NaCl in Buffer A (20 mM HEPES/NaOH pH 7.4, 0.5 mM EDTA, 1 mM DTT, 1 mM PMSF), then 0.22 μm filtered and loaded on a 20 ml-bed volume Q Sepharose Fast Flow column (Amersham Biosciences) previously equilibrated in Buffer A/150 mM NaCl. The chromatography was performed at 4°C using an Äkta purifier (Amersham Biosciences). The column was washed with five column volumes (CV) of equilibration buffer and eluted with a 10 CV linear gradient from 150 to 700 mM NaCl in Buffer A. Fractions of 2 ml were collected. Fractions active for cytochrome *c* release were pooled and concentrated 2.5-fold on a PEG bed. After centrifugation, the solution was loaded on a HiLoad 16/60 Superdex 200 (Amersham Biosciences), equilibrated in 20 mM HEPES/NaOH pH 7.4, 200 mM NaCl, 0.5 mM EDTA, 1 mM DTT, and 1 mM PMSF.

Production and purification of recombinant proteins

Bid cDNA was amplified using the polymerase chain reaction (PCR) from a HeLa cDNA library and cloned (*NcoI/BamHI*) in the pQE60 vector (QIAGEN) in frame with a C-terminal 6 × Histidine Tag. The cDNA encoding for Bcl-X_L was cloned (*SphI/SacI*) in the pQE30 vector (QIAGEN). Protein expression and purification were carried out according to the manufacturer's instructions. Most of Bid protein eluted at 60 mM imidazole from the Nickel NTA agarose (QIAGEN). Bcl-X_L eluted at 300 mM imidazole. Purified proteins were dialysed against 20 mM HEPES/KOH pH 7.4, 10% sucrose, 50 mM KCl, 1 mM DTT, 1 mM PMSF, aliquoted and stored at –80°C.

LacZ cotransfections

Rat1/MycER™ cells (5 × 10⁶) were electroporated in 10% FCS with 10 μg of pcDNA3.1/LacZ, plus 30 μg of pcDNA3.1 (Vec), 30 μg of pcDNA3.1/Bid-his (Bid) or 30 μg of pcDNA3.1/BidD60E-his (BidDE). Cells (2 × 10⁵) from each transfection were seeded in duplicate in six-well plates overnight in 10% FCS. The day after, cells were serum starved in 1% FCS for 24 h. The samples were treated with or without 100 nM 4-OHT for 16 h, washed in PBS and fixed with 0.25% glutaraldehyde in PBS for 15 min. Cells were stained with filtered X-Gal solution (0.2% X-Gal, 2 mM MgCl₂, 5 mM K₄Fe(CN)₆, 5 mM K₃Fe(CN)₆) for 16 h at 37°C. After washing with PBS, the number of blue cells was counted in each sample. The total number of cells was extrapolated by counting the number of cells present in 1 cm² of plate. The per cent of decrease of blue cells was derived by comparing the percentages of blue cells left in the samples coming from the same transfection, treated or not with 4-OHT. The experiment has been carried in duplicate.

Isoelectrofocusing

Rat1/MycER™ cells (2 × 10⁶) were serum starved for 24 h and then treated with or without 4-OHT for 8 h. Cell pellets were lysed directly in rehydration solution (8 M urea, 0.5% CHAPS, 0.2% DTT, 0.5% IPG buffer, 0.002% bromophenol blue). Rehydration and first dimension were carried using an Ettan IPGphor apparatus (Amersham Biosciences) according to the manufacturer's instructions. Focused 18-cm strips (3–10 pH interval) were equilibrated in denaturing solution (2% SDS, 50 mM Tris/HCl pH 8.8, 6 M urea, 30% glycerol, 0.002% bromophenol blue, 0.2% DTT). Thiol groups were blocked using 25 mg/ml of iodoacetamide according to the

manufacturer's instruction. A 7 cm of the strip corresponding to the pH interval 5–6 was cut out and loaded on a 15% SDS-PAGE.

Analysis of Bax conformational change

Cells were seeded onto glass coverslips in six-well plates and allowed to attach and grow for 24 h in DMEM/10% FCS. The medium was then replaced by DMEM/0.2% FCS for a further 24 h. c-MycER was activated by the addition of 4-OHT for 4 or 6 h. Control wells were treated with an equal volume of EtOH vehicle. After rinsing twice with cold PBS, cell monolayers were fixed by addition of 1% paraformaldehyde in PBS for 5 min at room temperature. Coverslips were washed three times in PBS and incubated with 6A7 anti-Bax monoclonal antibody (Trevigen) diluted 1/200 in PBS containing 500 µg/ml digitonin at 4°C overnight. After washing three times in PBS, bound antibody was detected by incubation with cy3-conjugated anti-mouse IgG (Amersham Biosciences) diluted 1/200 in 1% BSA/PBS. Coverslips were then washed three times in PBS, mounted with ProLong Antifade (Molecular Probes) and analysed by fluorescence microscopy (Zeiss, UK). The percentage of Bax-positive cells was quantified by counting a minimum of 500 cells in five randomly chosen fields for each treatment/time point. Positive cells showing a strong, punctate staining pattern are expressed as a percentage of the total number of cells analysed. Values represent the mean and variation of a typical experiment performed in duplicate.

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