Modulation of Bax mitochondrial insertion and induced cell death in yeast by mammalian protein kinase Cα

Rui D. Silva[a], Stéphen Manon[b], Jorge Gonçalves[c1], Lucília Saraiva[c2] and Manuela Côrte-Real[a]

[a]Molecular and Environmental Biology Centre, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal; [b]CNRS, UMR5095, Université de Bordeaux 2, 1 Rue Camille Saint-Saëns, 33077 Bordeaux, France; [c]REQUIMTE/CEQUP, Laboratório de Farmacologia[1] e Microbiologia[2], Faculdade de Farmácia, Universidade do Porto, Rua Aníbal Cunha, 4050-030 Porto, Portugal

Address correspondence to
Manuela Côrte-Real
Department of Biology, University of Minho
Campus de Gualtar,
4710-057 Braga
Portugal.
Telephone:+351 253604310
Fax: +351 253678980
Email: mcortereal@bio.uminho.pt

Abbreviations

PKCα, protein kinase Cα; ROS, reactive oxygen species; Cyt *c*, cytochrome c; PI, propidium iodide; H_2DCFDA, dichlorodihydrofluorescein diacetate.

Abstract

Protein kinase Cα (PKCα) is a classical PKC isoform whose involvement in cell death is not completely understood. Bax, a major member of the Bcl-2 family, is required for apoptotic cell death and regulation of Bax translocation and insertion into the outer mitochondrial membrane is crucial for regulation of the apoptotic process. Here we show that PKCα increases the translocation and insertion of Bax *c*-myc (an active form of Bax) into the outer membrane of yeast mitochondria. This is associated with an increase in cytochrome *c* (cyt *c*) release, reactive oxygen species production (ROS), mitochondrial network fragmentation and cell death. This cell death process is regulated, since it correlates with an increase in autophagy but not with plasma membrane permeabilization. The observed increase in Bax *c*-myc translocation and insertion by PKCα is not due to Bax *c*-myc phosphorylation, and the higher cell death observed is independent of the PKCα kinase activity. PKCα may therefore have functions other than its kinase activity that aid in Bax *c*-myc translocation and insertion into mitochondria. Together, these results give a mechanistic insight on apoptosis regulation by PKCα through regulation of Bax insertion into mitochondria.

Keywords

Apoptosis; Bax; Protein kinase C; yeast mitochondria

Introduction

Apoptosis is a form of programmed cell death with important roles in a wide variety of mammalian physiological processes and, when inappropriately controlled, is responsible for several pathologies. A crucial feature of mammalian apoptosis is the permeabilization of membrane organelles, namely mitochondria, and the release of apoptogenic factors that leads to activation of proteases responsible for cell death. The Bcl-2 family is critical for regulation of this permeabilization. The pro-apoptotic members of this family Bax and Bak are membrane multidomain proteins essential for the completion of apoptosis, since their deletion completely impairs this process [1]. Despite the importance of these proteins, the mechanisms by which they are regulated are not fully understood.

The pro-apoptotic function of Bax depends on its ability to translocate, oligomerize and insert into the mitochondrial membrane following stress [2-4]. Modulation of Bax can occur by phosphorylation, a post-translational modification. Indeed, it has been reported that phosphorylation of different Bax residues modulates its activity. Phosphorylation of ser184 by protein kinase B (Akt/PKB) and protein kinase Cζ (PKCζ) [5-7] promotes cell survival that is prevented by dephosphorylation by the protein phosphatase 2A [8]. Phosphorylation of ser163 by glycogen synthase kinase 3β (GSK3β) [9] and of thr167 by JNK and p38 kinase [10] lead to Bax activation and cell death. Bax can also be regulated by interaction with other proteins, thus preventing its translocation to mitochondria and hindering its cytotoxic effect. Bax-interacting proteins identified so far are, among others, Bcl-2 and its homologous proteins [11, 12], adenine nucleotide translocator [13], voltage-dependent anion channel protein [14], humanin [15], 14-3-3 [16], heat shock protein Hsp60 [17], PKCε [18], and Asc [19].

The PKC family is a multigene family of serine/threonine kinases with at least ten isoforms. They are classified into three subfamilies based on their structure and cofactors required for activation: the conventional or classical (α, βI, βII and γ), the novel (δ, ε, η, and θ) and the atypical (ζ and ι/λ) isoforms [20]. PKC isozymes are ubiquitously expressed, and PKCα, β, and δ are the most abundant isozymes in various tissues [21]. Although PKCs have a clear role in cell death, it has been a challenge to establish the relative contribution of the individual isoforms, owing to the different roles of PKC isoforms according to cell type and cellular localization [22]. Growing evidence indicates that PKC family members play important roles in regulating cell survival and apoptosis (for a review see 23) and their role in the modulation of Bcl-2 family has been the subject of increased attention.

Although several reports suggest a pro-survival role for PKCα, conflicting data indicating a pro-apoptotic function have been reported. In several cell lines, both depletion of PKCα or expression of a dominant negative form of PKCα lead to apoptosis induction [24-27]. PKCα phosphorylates Bcl-2 at serine 70, which is required for functional suppression of apoptosis in murine growth factor dependent cell lines [28]. Other reports show induction of apoptosis in the presence of PKCα. PKCα was shown to mediate activation of caspase-3 in renal proximal tubule cells [29] and to mediate Lamin B phosphorylation in HL60 cells [30]. In human prostate cancer cells, the presence of PKCα in non-nuclear membranes was associated with apoptosis, while its absence resulted in resistance to apoptosis [31]. In the same cell line, Tanaka and colleagues [32] showed that p38 MAPK mediates PKCα-induced apoptosis and that PKCα leads to dephosphorylation and inactivation of the survival kinase AKT, probably mediated by protein phosphatase 2A.

While studies of mammalian cell lines lacking specific components of the apoptotic machinery or isoforms of the PKC signalling cascade have contributed substantially to our understanding, it would be almost impossible to use cells with all the relevant genes silenced or knocked out. Yeast lacks obvious homologues of many key mammalian apoptotic regulators, including the Bcl-2 family, and it has therefore been used as an "in vivo" system to study several of these apoptotic regulators. Indeed, when many of these proteins are expressed in yeast, they conserve their functional and molecular effect at several cellular levels, namely at the mitochondria (for a review see 33 and 34). In the present study, we used yeast to investigate the role of PKCα in the regulation of the pro-apoptotic Bcl-2 family protein Bax. Our results demonstrate that PKCα increases the translocation and insertion of Bax *c*-myc (a form of Bax in the active conformation) into the yeast mitochondria by a mechanism independent of the PKCα kinase activity.

Material and methods

Yeast strains, plasmids and growth conditions

The wild-type haploid *Sacharomyces cerevisiae* strain CG379 (*mat* α *ade5 his7-2 leu2-112 tryp1-289α ura3-52* [*kil-O*], yeast genetic stock center, University of California, Berkeley, USA) was used throughout this study. For PKCα expression, the bovine PKCα was cloned into the YEp51 yeast expression plasmid (*LEU2*) under the control of a *GAL10* promoter. For Bax *c*-myc expression, the isoform α of the human *bax* gene was chemically synthesized with yeast codon bias and fused to the *c*-myc epitope cloned into the centromeric plasmid pCM184 (*TRP1*) under the control of a Tet-Off promoter (repressed by the addition of doxycycline) as described in [35].

The GFP-Atg8p construction (as described in 36) is in the pRS416 plasmid under control of the endogenous Atg8p promoter. Site directed mutagenesis of bovine PKCα was done using the QuickChange method (Stratagene) with the primers GAGCTGTACGCCATCCGTATCCTGAAGAAGGACGTGG and CCACGTCCTTCTTCAGGATACGGATGGCGTACAGCTC. The mutant PKCα was sequenced to verify the introduction of the desired substitution. pCLbGFP (*URA3)*, encoding GFP fused to the mitochondrial presequence of citrate synthase (mt-GFP) under the control of the *GAL1*/*10* promoter was used to monitor mitochondrial morphology [37]. Expression of PKCα and Bax *c*-myc was done sequentially. Yeast cells were first grown in synthetic medium (0.17% yeast nitrogen base, 0.5% ammonium sulphate, 0.1% potassium phosphate and 80 μg/ml of all auxotrophic requirements except leucine and tryptophan, pH 5.5) with 2% glucose, 10 μg/ml of doxycycline to repress Bax *c*-myc expression. Cells were then transferred to synthetic medium with 2% galactose, 1% raffinose, 3% glycerol and 10 μg/ml doxycycline to induce PKCα expression and grown to an OD at 640 nm of 1.0. Finally, cells were transferred to synthetic medium with 2% galactose without doxycycline and diluted to an OD at 640 nm of 0.1 to induce both proteins. Cells were collected at different times and processed further. All incubations were performed at 30ºC, 200 r.p.m.

Cell death assay and effect of PKCα inhibitors on cell death

For cell death assays, samples were harvested at the indicated times, the number of cells counted, and 100 cells plated in YPD plates with 10 μg/ml of doxyxycline. Plates were incubated at 30 ºC and the number of colonies counted after 48 hours. Data represent the number of c.f.u. at time *t*

divided by the number of c.f.u. in the control (cells carrying the empty vector of PKCα and grown in the presence of doxycycline) for the same time.

The PKCα inhibitors Gö 6976 (Sigma) and Ro 32-0432 (Sigma) were prepared in dimethyl sulfoxide (DMSO) at a final concentration of 1 mM. Cells were transferred to synthetic medium with 2% galactose without doxycycline and diluted to an OD at 640 nm of 0.1, to express both proteins, and DMSO, Gö 6976 or Ro 32-0432 were added to the culture at a final concentration of 0.1% and 1μM, respectively. Cell survival was measured by platting efficiency as described above.

PI staining and ROS production

Propidium iodide (PI) staining and ROS production were monitored by flow cytometry. Labelling with PI was performed by incubating 10^6 cells in culture medium containing 2 μg/ml of PI (Sigma) for 15 minutes. ROS production was monitored in cells preserving plasma membrane integrity by double staining with PI and dichlorodihydrofluorescein diacetate (H_2DCFDA; Molecular Probes). Conversion of H_2DCFDA to DCF was analysed in PI negative cells. About 10^6 cells were incubated in culture medium containing 40 μg/ml H_2DCFDA for 45 minutes at 30ºC. 2 μg/ml of PI was added after 30 minutes of incubation. Flow cytometric analysis was performed in an Epics® XL™ (BeckmanCoulter) flow cytometer equipped with an argon-ion laser emitting a 488 nm beam at 15 mW. Green fluorescence was collected through a 488 nm blocking filter, a 550 nm long-pass dichroic and a 525 nm band-pass filter. Red fluorescence was collected through a 560 nm short-pass dichroic, a 640 nm long-pass, and another 670 nm long-pass filter. 20,000 cells were analysed per sample at low flow rate. Data were analysed by WinMDI 2.8 software.

Mitochondrial network fragmentation

Cells expressing PKCα, Bax *c*-myc, PKCα and Bax *c*-myc or none of the proteins (control) were co-transformed with pCLbGFP. Cells were collected at different times and fragmentation of the mitochondrial network evaluated by epifluorescence microscopy. At least 150 cells per sample were classified. In this set of experiments uracil was also omitted from the growth medium.

Western blot analysis

Cells extracts were prepared as described in [38]. Protein lysates were separated on 12.5% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes (hybond-P; Amersham). The membranes were blocked with 5% non-fat milk in phosphate-buffered saline containing 0.05% Tween 20 for 30 minutes at room temperature. Membranes were then incubated overnight at 4ºC with primary antibodies directed against human Bax (rabbit polyclonal, 1:10000; Sigma), bovine PKCα (mouse monoclonal, 1:2000; Upstate), yeast phosphoglycerate kinase (mouse monoclonal, 1:10000; Molecular Probes), yeast Atg8p (rabbit polyclonal, 1:200; Santa Cruz Biotechnology), GFP (mouse monoclonal, 1:3000; Roche Applied Science) or yeast Por1p (mouse monoclonal, 1:5000; Molecular Probes). Peroxidase-coupled secondary antibodies were from Jackson ImmunoResearch Laboratories (1:10000) and membranes were incubated for 1 hour at room temperature. Peroxidase activity was revealed by chemioluminescence (Immobilon Western, Millipore).

Mitochondria preparation, carbonate treatment and cyt *c* content analysis

Mitochondria were isolated by differential centrifugation from zymolyase-treated cells, as described previously [39]. For carbonate and Triton X-100 extraction, 1 mg of protein from

isolated mitochondria was incubated in the presence of 0.1 M Na_2CO_3 (pH 10.0) or Triton X-100 for 15 minutes and centrifuged for 15 minutes at 105,000g. The presence of Bax *c*-myc in the pellet and the supernatant was verified by western blot.

Assessment of cyt *c* content was measured by redox spectra of isolated mitochondria essentially as described previously [40]. Differential spectra of the reduced (sodium dithionite) minus oxidized (potassium ferricyanide) extracts were recorded on a double-beam/double wavelength spectrophotometer (Aminco DW2000). The maxima absorption for cyt *b* and for cyt *c+c1* used were 561 and 550 nm, respectively. The cyt *c*/cyt *b* ratio was always used to normalize the total protein content from the different samples.

Immunoprecipitation and detection of phosphorylated serines

Immunoprecipitation was performed using the IP50 kit from Sigma as described in [41]. Briefly, cells were ressuspended in buffer (Tris-malate 10 mM, Mannitol 0.6 M, EGTA 1 mM, pH 6.7) supplemented with a mixture of protease (complete protease inhibitor cocktail tablets; Roche Applied Science) and phosphatase (PhosSTOP phosphatase inhibitor cocktail tablets; Roche Applied Science) inhibitors. Cells were broken mechanically by vortexing with glass beads, after which 100 μl of 10x lysis buffer (IP50 kit; Sigma) was added to 1 ml of cell suspension and incubated at 4ºC during 1 hour. 2 μg of monoclonal anti-Bax antibody (2D2; Sigma) was added, and the lysate incubated overnight at 4°C. Protein G-coupled agarose beads (IP50; Sigma) were added and incubated for 6 hours. Washing and recuperation of the samples were done following the manufacturer's instructions. Identical samples were loaded in parallel onto two SDS-PAGE gels and blotted. One was probed with a monoclonal anti-phosphoserine antibody (7F12; 1:5000; ALEXIS Biochemicals), and the other was probed with a polyclonal anti-Bax antibody.

[^{32}P] Phosphate labelling

For phosphate labelling, expression of PKCα and Bax *c*-myc were done in a low phosphate medium [42]as in [41]. Briefly, ^{32}P phosphate (0.25mCi/ml) was added 6 hours after Bax *c*-myc induction, and cells collected after 2 hours. Bax *c*-myc was immunoprecipitated using the protocol described above, loaded onto two SDS-PAGE gels and blotted. One membrane was exposed to autoradiography film, and the other was probed with a polyclonal anti-Bax antibody.

Results

Mammalian PKCα enhances Bax *c*-myc-induced cell death without disturbing plasma membrane integrity

Bax α needs to be activated in order to induce organelle membrane permeabilization, and thus trigger apoptosis. So, expression of native human Bax in yeast, a system that lacks several homologues of mammalian apoptotic regulators, has no effect on yeast viability [43]. Therefore, in order to study the effect of mammalian PKCα in the regulation of Bax using yeast, we expressed a form of Bax in the active conformation that is cytotoxic for this organism (Bax *c*-myc) [35]. Our results show that cell death induced by expression of Bax *c*-myc in yeast is increased by co-expression with PKCα (Fig. 1A). This increase in cell death is not accompanied by loss of plasma membrane integrity, measured by PI staining (Fig. 1B). The maintenance of plasma membrane integrity suggests that, as already described for expression of Bax *c*-myc alone [44], the death process in cells co-expressing PKCα and Bax *c*-myc is a regulated event.

Expression of PKCα enhances Bax *c*-myc-induced ROS production, cyt *c* release and mitochondrial network fragmentation

Yeast cell death induced by Bax *c*-myc is usually accompanied by several functional and biochemical markers such as ROS production [38, 45], cyt *c* release [40], and fragmentation of the mitochondrial network [44]. The effect of PKCα in Bax *c*-myc ROS production, cyt *c* release, and fragmentation of the mitochondrial network was evaluated in cells co-expressing PKCα and Bax *c*-myc and compared to cells expressing Bax *c*-myc alone. ROS production increases in cells co-expressing PKCα and Bax *c*-myc (Fig. 2A). In addition, cells co-expressing PKCα and Bax *c*-myc have a lower cyt *c* content (Fig. 2B) and increased mitochondrial network fragmentation (Fig. 2C,D). These results indicate that PKCα enhances the cytotoxic effects of Bax *c*-myc expression in yeast cells.

Co-expression of PKCα and Bax *c*-myc stimulates autophagy

An increased amount of Atg8p has been observed in yeast following nitrogen starvation, rapamycin treatment or Bax *c*-myc expression. The increase in the amount of this autophagic protein is considered one of the typical markers of autophagy induction [44, 46]. In order to determine whether PKCα also interferes with Bax *c*-myc-induced autophagy, Atg8p expression was evaluated by western blot in cells expressing PKCα, Bax *c*-myc, co-expressing PKCα and Bax *c*-myc, and in control cells. It has been previously shown that Bax *c*-myc stimulates Atg8p expression [44]. Accordingly we were also able to detect a two-fold increase in Atg8p expression after Bax *c*-myc expression. However we did not detect any difference in Atg8p expression between control cells and PKCα expressing cells (Fig. 3A). In cells co-expressing both proteins there was a seven-fold increase in Atg8p expression, indicating that autophagy is increased.

In order to further confirm that the higher Atg8p expression detected was associated to autophagy induction we also monitored the level of Atg8p that is delivered into the vacuole. For this purpose a GFP-Atg8p fusion was also expressed in our transformed cells. When this fusion is delivered into the vacuole the Atg8p is rapidly degraded by vacuolar hydrolases while free GFP is not degraded. So, accumulation of the GFP moiety reflects delivery of Atg8p into the vacuole and therefore the level of autophagy induction [36]. Cells expressing the GFP-Atg8p fusion displayed an accumulation of free GFP corresponding to 7% and 15% of the total GFP, when Bax *c*-myc is expressed, or PKCα and Bax *c*-myc are co-expressed, respectively. These observations indicate a higher delivery of Atg8p into the vacuole and confirmed a higher autophagy level when both proteins are co-expressed (Fig. 3B). In control cells and in cells expressing PKC no accumulation of free GFP was detected (Fig. 3B).

PKCα increases the insertion of Bax *c*-myc into the mitochondrial membrane

When expressed in yeast cells, Bax *c*-myc translocates to the mitochondria and inserts into the mitochondrial membrane, leading to several downstream events described above. The presence of PKCα and Bax *c*-myc in whole cell extracts and in the mitochondrial fraction was verified by western blot. Both proteins were expressed in yeast cells, and there was an accumulation of Bax *c*-myc (about 2.3 times) in cells co-expressing PKCα (Fig. 4A). The possibility that this increase could be due to interference by PKCα with the promoter of Bax *c*-myc was unlikely. However we did check this possibility by expressing PKCα with Bcl-xL, another protein with mitochondrial localization, under control of the same expression system (pCM184) used for Bax *c*-myc expression. We could confirm that there was no effect on the expression of Bcl-xL, thus ruling out the hypothesis of a non-specific effect of PKCα on the promoter of the plasmid used

for Bax *c*-myc expression (data not shown). Analysis of the mitochondrial fraction confirmed the translocation of Bax *c*-myc to the mitochondria as revealed by an increase in the amount of Bax *c*-myc in the mitochondrial fraction (about 4.6 times) when PKCα is co-expressed (Fig. 4A). This increase is much higher than that observed in whole cell extracts, indicating that the accumulation of Bax *c*-myc observed under co-expression conditions occurs preferably at mitochondria. In fact, the accumulation observed in whole cell extracts might be due to a higher translocation to mitochondria since Bax *c*-myc is more protected from degradation in the lipidic environment of the outer mitochondrial membrane.

PKCα could lead to an increase in the actual insertion of Bax *c*-myc into the mitochondrial membrane or only to an enhanced association. Isolated mitochondria from cells expressing Bax *c*-myc or co-expressing PKCα and Bax *c*-myc were therefore treated with Na_2CO_3 or Triton X-100 to remove loosely bound or inserted proteins, respectively. Bax *c*-myc was partially insensitive to carbonate treatment but sensitive to Triton X-100, showing that it is mainly inserted into the mitochondrial membrane (Fig. 4B). The maintenance of the ratio between associated and inserted Bax *c*-myc in yeast cells expressing Bax *c*-myc and co-expressing PKCα and Bax *c*-myc shows that the higher translocation of this protein is associated with a higher insertion.

Analysis of the mitochondrial fraction also revealed the presence of PKCα in mitochondria independently of the co-expression with Bax *c*-myc (Fig. 4C).

PKCα does not alter Bax *c*-myc phosphorylation in yeast

Arokium *et al*. [41] showed that human Bax α is phosphorylated in yeast cells and mutation of possible phosphorylation serine sites in the protein enhances the ability of Bax α to insert into

the mitochondria and to induce cyt *c* release. Interestingly, we were not able to detect phosphorylation of Bax *c*-myc either in cells expressing Bax *c*-myc or co-expressing PKCα and Bax *c*-myc, using an antibody previously shown to detect Bax α with phosphorylated serines [5]. As a positive control, Bax α immunoprecipitated from yeast cells was used (Fig. 5A). To confirm that Bax *c*-myc is not phosphorylated in yeast cells, in vivo radioactive labelling was performed. Phosphorylation of Bax *c*-myc was not detected, with or without expression of PKCα (Fig. 5B). These results indicate that the higher insertion of Bax *c*-myc in the presence of PKCα, and its associated effect described above is not related to an alteration of the Bax *c*-myc phosphorylation state.

PKCα kinase activity is not involved in enhancing the effect of Bax *c*-myc

To study the relation between PKCα kinase activity and the enhancement of the events induced by Bax *c*-myc, the viability of yeast cells expressing both proteins was assessed in the presence of two PKC inhibitors, Gö 6976 and Ro 32-0432. The concentration of both inhibitors tested was selected using a yeast phenotypic assay as described in [47]. Curiously, the results obtained showed that these inhibitors have no effect on the viability of yeast cells expressing both proteins (Fig. 6A). A catalytically inactive mutant of PKCα (the PKCα^{K368R}) was also co-expressed with Bax *c*-myc and its effect on cell viability compared with that obtained with wild-type PKCα. In this mutant, a lysine residue in the ATP-binding site of the protein was replaced with an arginine, leading to the loss of phosphorylation activity [48]. Co-expression of PKCα^{K368R} and Bax *c*-myc was confirmed by western blot (data not shown). Co-expression of PKCα^{K368R} or PKCα with Bax *c*-myc had similar effects in cell viability (Fig. 6B). These results indicate that the effect of PKCα on Bax *c*-myc expressing yeast cells does not depend on PKCα kinase activity.

Discussion

In previous studies, we took advantage of yeast to study the role of mammalian PKC isoforms on the regulation of apoptosis and the Bcl-2 anti-apoptotic protein Bcl-xL [49]. In the present work, yeast was used to study the role of PKCα on the regulation of Bax, one of the most important proteins in the mitochondrial apoptotic cascade. We assessed whether PKCα, a member of the classical PKC subfamily, modulates Bax without the interference of other Bcl-2 family proteins and PKC isoforms by expressing these two proteins in yeast.

In this work, we show that PKCα regulates the effect of Bax *c*-myc, an active form of Bax α, by increasing its translocation and insertion into the outer mitocondrial membrane. This leads to an enhancement of other Bax *c*-myc induced-downstream events in yeast cells, such as loss of viability, ROS production, mitochondrial network fragmentation, cyt *c* release, and higher Atg8p expression and vacuolar delivery. In contrast, no increase in loss of plasma membrane integrity was detected. Several reports show that autophagy is activated following Bax *c*-myc expression [38, 44]. These authors showed that autophagy was not responsible for the loss of plating efficiency but rather played a minor role in maintaining cell survival. However, they found that mitophagy is required for regulated loss of cell survival since absence of Uth1p (a protein required for mitophagy) led to a higher percentage of PI-positive cells. Here, the enhancement of Bax *c*-myc induced cell death by PKCα is unlikely related to an inhibition of autophagy, since there is an accumulation of Atg8p, a higher delivery of this protein to the vacuole and no increase in the percentage of PI positive cells. The higher amount of Atg8p and the higher

vacuolar delivery detected in cells co-expressing PKCα and Bax *c*-myc is likely due to the observed higher translocation of Bax *c*-myc to mitochondria, which in turn results in higher autophagy induction.

A great benefit of studies with animal tissue cultures is the possibility of determining the final cellular effect of a given modulator. However, it is difficult to study the specific effect of such modulator on a particular protein. The effect of PKCα on other Bcl-2 family proteins such as Bax is difficult to study in an environment where other PKCα-regulatable apoptosis modulators are present. By expressing PKCα and Bax *c*-myc in yeast, we were able to study the regulation of Bax *c*-myc by PKCα in the absence of all other Bcl-2 family proteins. We found a mitochondrial localization of PKCα, higher insertion in Bax *c*-myc on the outer mitochondrial membrane and higher cell death in cells co-expressing PKCα. Previous studies with mammalian cells have uncovered a mitochondrial localization of PKCα [50, 51]. However, it was linked with an increase of cell survival. Whether the presence of PKCα in the mitochondria is essential for enhancement of Bax *c*-myc induced cell death in yeast is unknown.

PKCα regulates several apoptotic proteins, as well as proteins upstream of the apoptotic cascade, through phosphorylation. Therefore, it would be reasonable to consider that PKCα regulates Bax *c*-myc through phosphorylation. It was surprising to find that the presence of PKCα does not alter the Bax *c*-myc phosphorylation state. In fact, phosphorylated Bax *c*-myc is not detected in yeast, in contrast with what was previously described for Bax α [41, this study]. It is possible that the conformational changes induced by the *c*-myc epitope or the insertion of Bax *c*-myc in the outer mitochondrial membrane protect target residues from phosphorylation. Our data clearly demonstrate that the enhancing effect of PKCα on Bax *c*-myc is not mediated by phosphorylation. In fact, the kinase-dead PKCα^{K368R} mutant, has the same effect on the increase

of Bax *c*-myc induced cell death as the wild-type PKCα. Consistently, the PKC inhibitors used in this study had no effect on Bax *c*-myc induced cell death in cells co-expressing Bax *c*-myc and PKCα. This shows that the kinase activity of PKCα is not necessary for the enhancement of Bax *c*-myc induced cell death and that a phosphorylation cascade is not involved in this process. It has previously been shown that PKCα enhances phosphorylation of Bcl-xL in yeast, abolishing its anti-apoptotic activity [49]. Here we show that PKCα also has a pro-apoptotic role in the modulation of Bax. However, this role is independent of its kinase activity, in contrast with the pro-apoptotic role observed for the modulation of Bcl-xL. It was reported that PKCε interacts with Bax, sequestering it in the cytosol. It is possible that a similar interaction between Bax *c*-myc and PKCα exists in this compartment or even at mitochondria. However, we could not detect it by immunoprecipitation (data not shown). The present study only focused on the regulation of Bax *c*-myc by PKCα. However we expect that isoforms from other PKC sub-families may regulate Bax differently. Actually, specific modulation by distinct PKC isoforms of the Bcl-2 protein family member Bcl-xL has already been reported [49].

In conclusion, our findings show that PKCα has a pro-apoptotic effect on Bax *c*-myc, increasing Bax *c*-myc induced cell death, translocation and insertion of Bax *c*-myc into the outer mitochondrial membrane, and enhances several other cellular events associated with Bax *c*-myc-induced death. We therefore propose a model where PKCα aids in the translocation and/or the insertion of Bax *c*-myc into the outer mitochondrial membrane by a still unknown mechanism, subsequently leading to an increase in cyt *c* release, ROS production, mitochondrial network fragmentation and cell death. Furthermore, an increase in the autophagic process allows the maintenance of a regulated form of cell death. This work together with our previous data on specific modulation of apoptosis and Bcl-xL phosphorylation by distinct mammalian PKC

isoforms [49] further reinforces the yeast model to study the regulation of Bcl-2 family proteins by PKC isoforms. Finally, a mechanistic insight on apoptosis regulation by PKCα through regulation of Bax insertion into mitochondria is provided.

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Figure legends

Figure 1

PKCα enhances Bax *c*-myc induced cell death in yeast without disturbing plasma membrane integrity. (A) Percentage of cell survival evaluated by c.f.u. About one hundred cells expressing PKCα, Bax *c*-myc, PKCα and Bax *c*-myc or none of the proteins (control) were taken at different times, plated and the number of c.f.u. evaluated. 100% survival corresponds to the number of c.f.u. obtained with the control for each time point. Data are the mean ± s.e.m. of five independent experiments. (B) Percentage of cells displaying loss of plasma membrane integrity evaluated by PI staining. Cells expressing PKCα, Bax *c*-myc, PKCα and Bax *c*-myc or none of the proteins (control) were collected at different times and the percentage of PI-positive cells was evaluated by flow cytometry. Data are the mean ± s.e.m. of three independent experiments. Significant differences obtained between Bax *c*-myc expression and PKCα and Bax *c*-myc co-expression are indicated by **P*<0.05; **0.01>*P*>0.001; ****P*<0.001 (unpaired Student's *t*-test).

Figure 2

Expression of PKCα enhances Bax *c*-myc-induced ROS production, cyt *c* release and mitochondrial network fragmentation in yeast. (A) ROS production was evaluated by monitoring the conversion of H_2DCFDA to DCF by flow cytometry. Results are expressed as ratio values estimated by dividing the mean fluorescence intensity of each sample by the mean fluorescence intensity of the control cells for the same time. Data are the mean ± s.e.m. of five independent experiments. (B) Cyt *c* content in mitochondria assessed by redox spectra analysis of isolated mitochondria from cells expressing Bax *c*-myc (full line) and cells co-expressing PKCα and Bax

c-myc (dashed line). The values of the cyt *c*/cyt *b* ratios are indicated in the image. (C) Percentage of cells displaying fragmentation of the mitochondrial network. Cells expressing mt-GFP and expressing PKCα, Bax *c*-myc, PKCα and Bax *c*-myc or none of the proteins (control) were collected at different times and fragmentation of the mitochondrial network was evaluated by epifluorescence microscopy. Data are the mean ± s.e.m. of three independent experiments; means correspond to counts of at least 150 cells per sample. (D) Fluorescence microscopy images of cells expressing mt-GFP and expressing PKCα, Bax *c*-myc, PKCα and Bax *c*-myc or none of the proteins (control) after 6 hours. Significant differences between Bax expression and co-expression of PKCα and Bax *c*-myc are indicated by **P*<0.05; **0.01>*P*>0.001; ****P*<0.001 (unpaired Student's *t*-test).

Figure 3

Co-expression of PKCα and Bax *c*-myc increases the level of autophagy. (A) Detection of Atg8p expression in whole cell extracts of control cells and cells expressing PKCα, Bax *c*-myc and co-expressing PKC-α and Bax *c*-myc, after 10 hours. Pgk1p was used as loading control. The amount of Atg8p was quantified by densitometry analysis of non saturated immunoblots. All values were normalised to the loading control. (B) Detection of free GFP generated from the GFP-Atg8p fusion protein in whole cell extracts of cells expressing this fusion and expressing PKCα, Bax *c*-myc and co-expressing PKC-α and Bax *c*-myc, after 14 hours. Pgk1p was used as loading control. The amount of GFP was quantified by densitometry analysis of non saturated immunoblots and the values showed are the percentage of the GFP in the cells that is not fused to Atg8p.

Figure 4

PKCα increases the translocation and insertion of Bax *c*-myc into the mitochondria. (A) Detection of PKCα and Bax *c*-myc in whole cell extracts and in the mitochondrial fraction. Pgk1p and Por1p were used as loading controls for the whole cell extracts and mitochondrial fraction, respectively. The amount of Bax *c*-myc was quantified by densitometry analysis of non saturated immunoblots. All values were normalised to the loading control. (B) Mitochondria isolated from cells expressing Bax *c*-myc only and co-expressing PKCα and Bax *c*-myc were treated with Na_2CO_3 or Triton X-100 to remove loosely bound or inserted proteins, respectively. (C) Detection of PKCα in the mitochondrial fraction. Por1p was used as loading control.

Figure 5

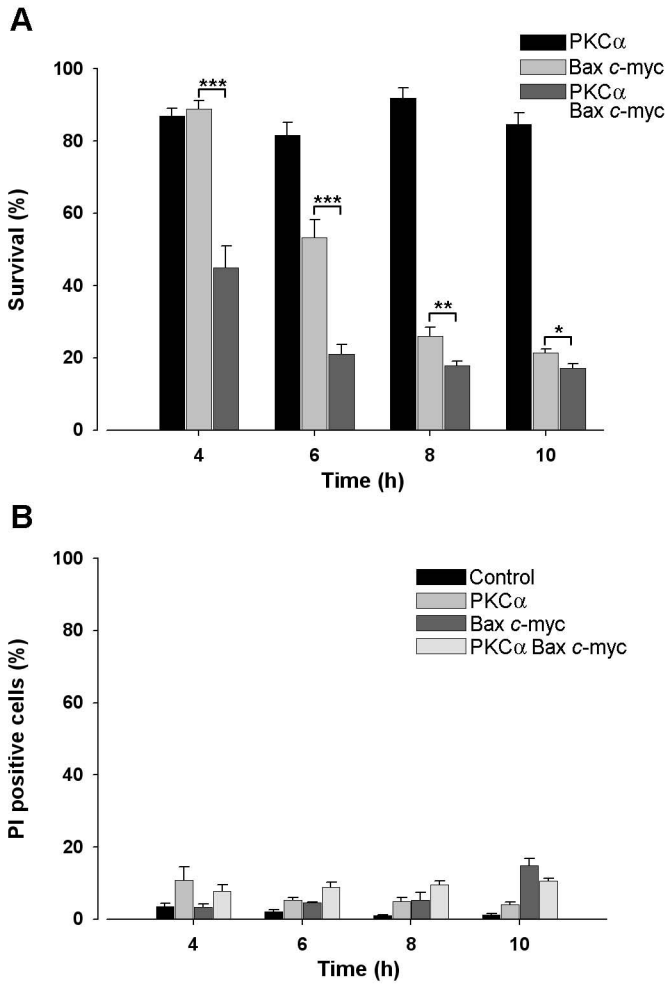
Bax *c*-myc is not phosphorylated in yeast. Phosphorylation of Bax *c*-myc was evaluated by using an anti-phosphorylated serine antibody (A) and by [^{32}P] phosphate labelling (B). (A) The search for phosphorylated serine residues in Bax *c*-myc was performed after 14 hours of induction. Membranes were probed with a monoclonal anti-phosphoserine antibody (7F12) and with a polyclonal anti-Bax antibody. As a positive control, immunoprecipitated Bax α (a phosphorylatable form of Bax in yeast) from yeast cells expressing Bax α, was also probed with the anti-phosphoserine antibody. (B) [^{32}P] phosphate labelling of cells grown in a low phosphate medium. Expression of Bax *c*-myc was induced by removal of doxycycline, ^{32}P phosphate (0.25mCi/ml) was added 6 hours later and cells were further incubated for 2 hours. Membranes were revealed by autoradiography or with a polyclonal anti-Bax antibody.

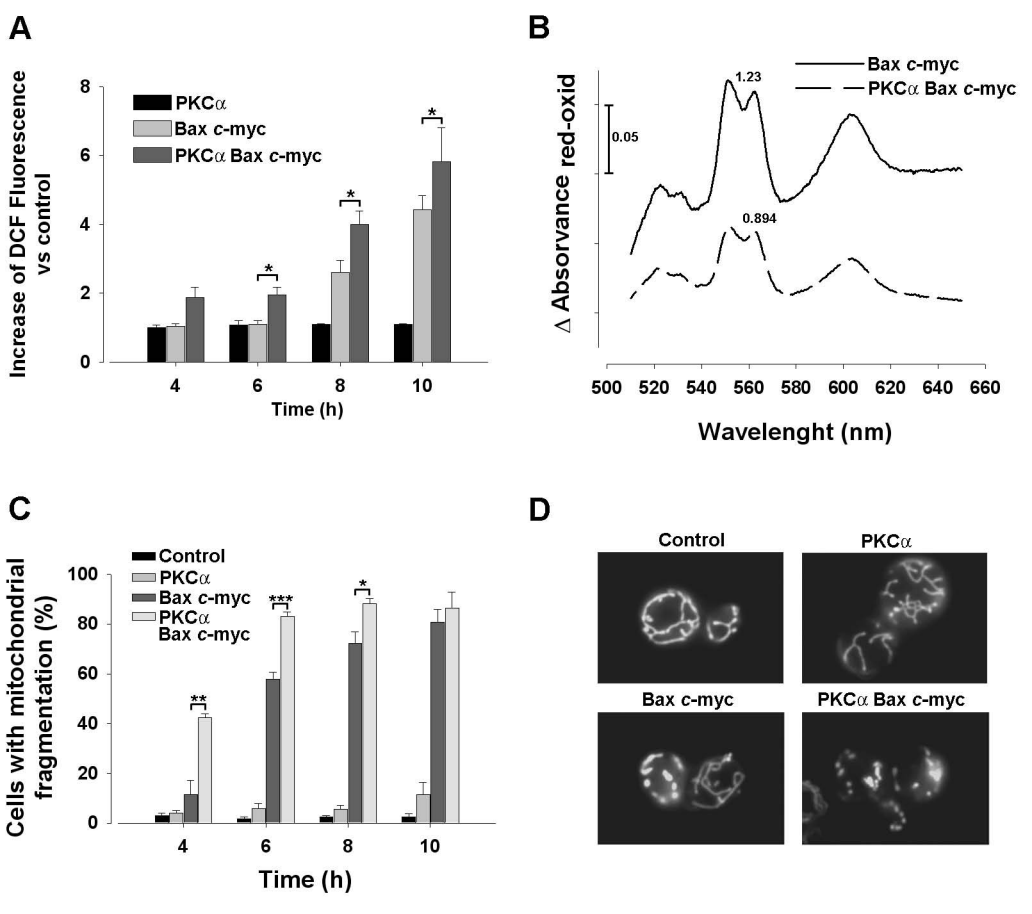
Figure 6

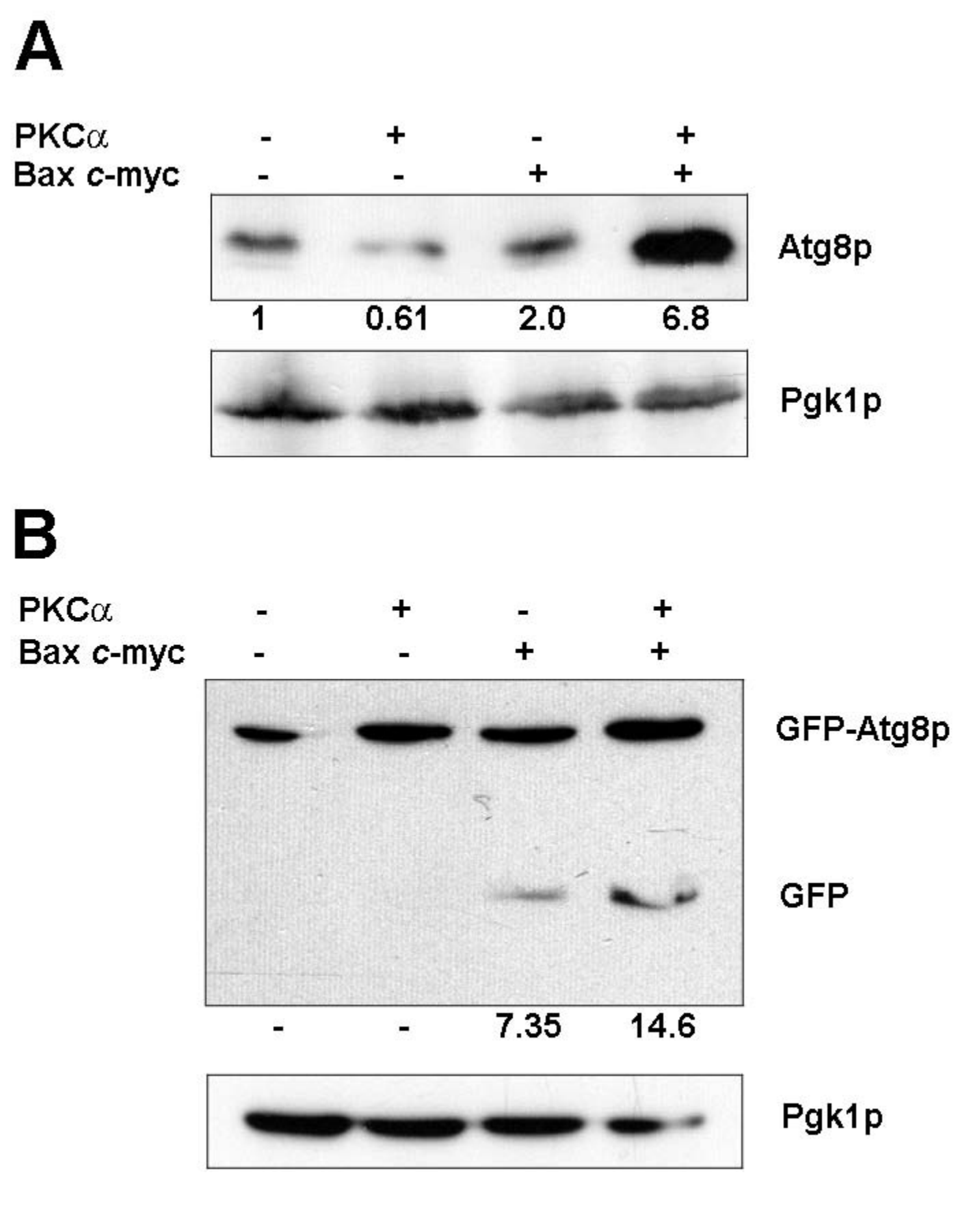
The effect of PKCα is independent of its kinase activity. (A) Effect of PKC inhibitors Gö 6976 and Ro 32-0432 in the stimulation of Bax *c*-myc induced cell death by PKCα. Survival of cells co-expressing PKCα and Bax *c*-myc in the absence or presence of the inhibitor was evaluated by c.f.u.. 100% survival corresponds to the number of c.f.u. obtained for time 0 (hours). (B) Comparison of survival percentage between cells co-expressing PKCα and Bax *c*-myc or co-expressing PKCα^{K368R} and Bax *c*-myc after 6 and 8 hours. Survival was evaluated by c.f.u. and 100% survival corresponds to the number of c.f.u. obtained with the control (cells expressing none of the proteins) for each time. Data are the mean ± s.e.m. of at least three independent experiments. Two-way ANOVA analysis revealed no significant effect on cell death of both PKC inhibitors and of the single point mutation K368R in PKCα.

Figure 7

Proposed model for PKCα regulation of Bax *c*-myc induced cell death. PKCα in the cytosol, mitochondria or in both compartments directly or indirectly increases translocation and/or insertion of Bax *c*-myc to mitochondria by an unknown mechanism leading to an increase in cyt *c* release, ROS production and mitochondrial network fragmentation. These alterations lead to an increase in autophagy that regulates the mode of cell death.


A
PKCα
Bax c-myc
PKCα Bax c-myc
Survival (%)
100
80
60
40
20
0
***
***
**
*
4
6
8
10
Time (h)
B
Control
PKCα
Bax c-myc
PKCα Bax c-myc
PI positive cells (%)
100
80
60
40
20
0
4
6
8
10
Time (h)


A
Increase of DCF Fluorescence vs control
PKCα
Bax c-myc
PKCα Bax c-myc
Time (h)
B
Bax c-myc
PKCα Bax c-myc
1.23
0.894
0.05
Δ Absorvance red-oxid
Wavelenght (nm)
C
Control
PKCα
Bax c-myc
PKCα Bax c-myc
Cells with mitochondrial fragmentation (%)
Time (h)
D
Control
PKCα
Bax c-myc
PKCα Bax c-myc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PKCα - + - +
Bax c-myc - - + +
Atg8p
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Pgk1p
B
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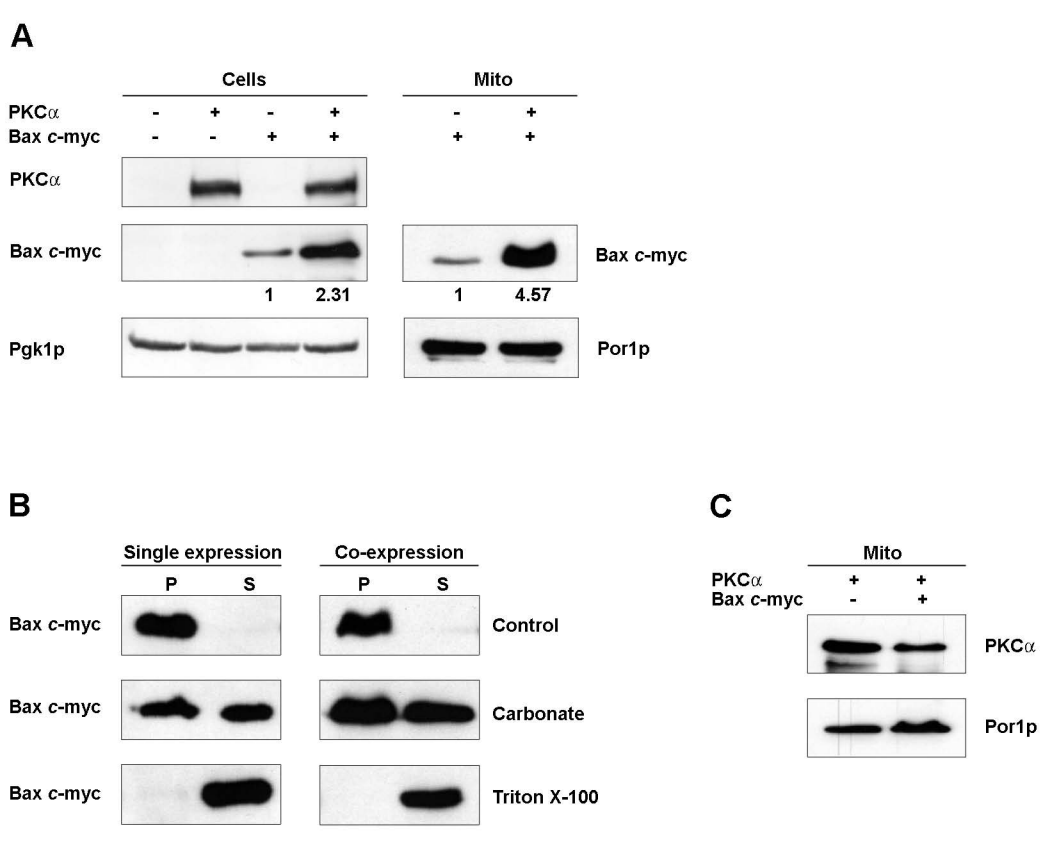
GFP-Atg8p
GFP
- - 7.35 14.6
Pgk1p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1
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Pgk1p
Por1p
B
Single expression
Co-expression
P
S
P
S
Bax c-myc
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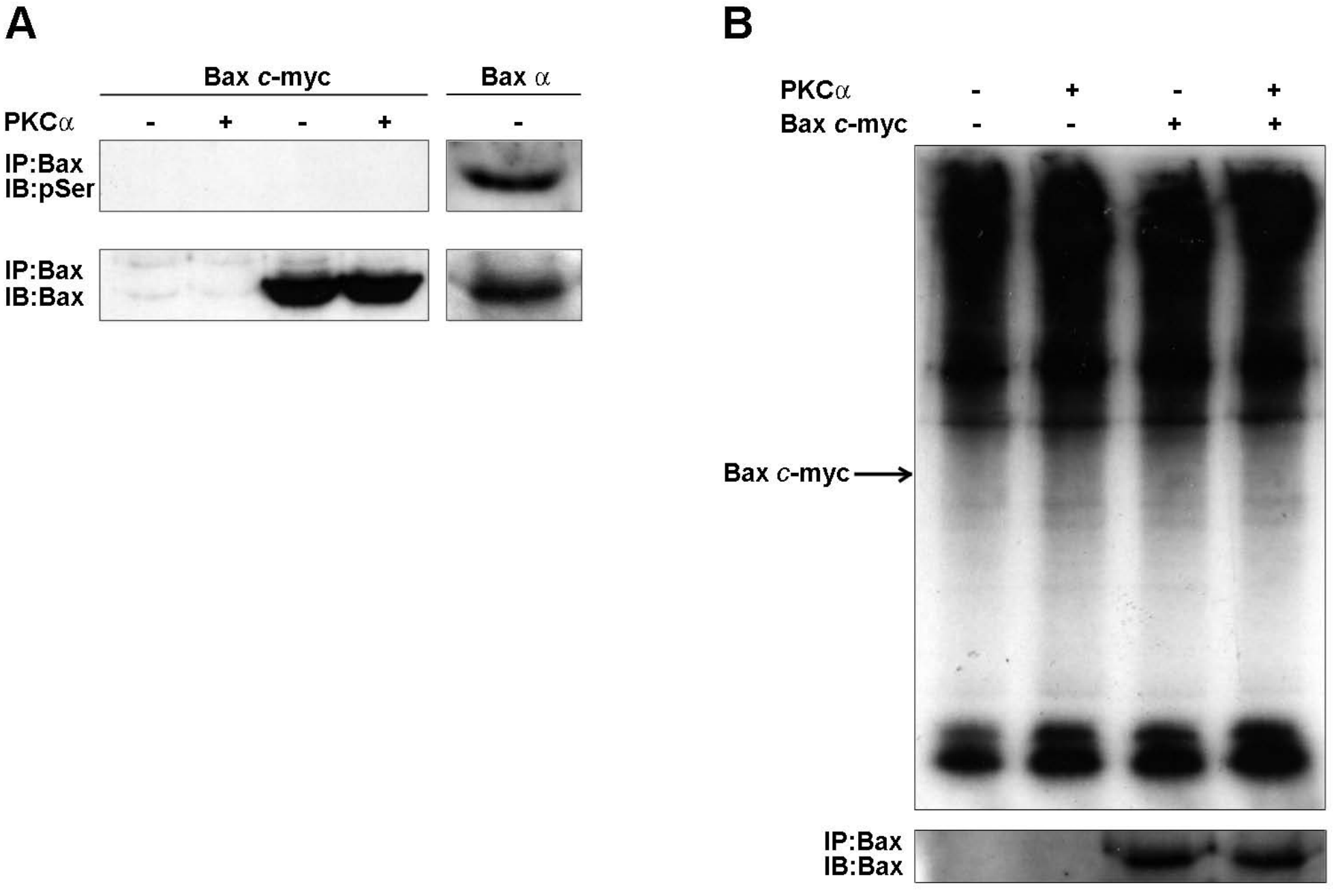
Por1p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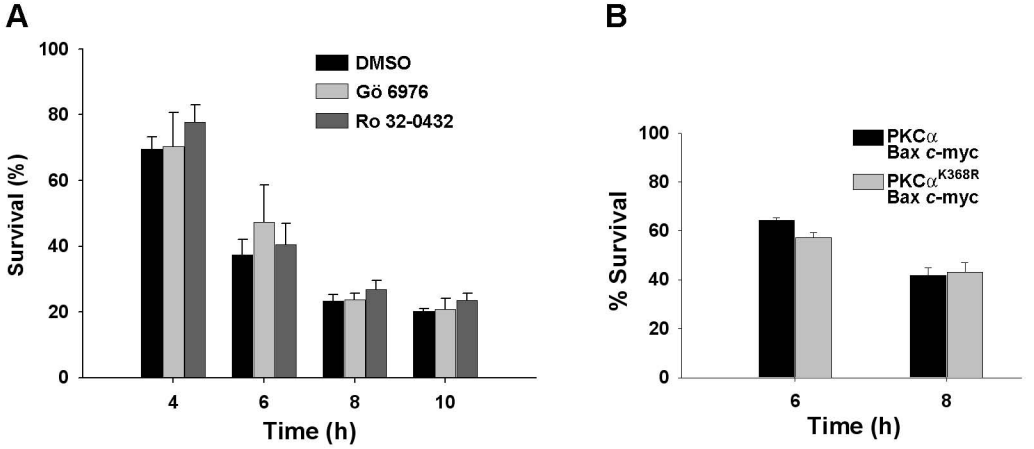
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100
80
60
40
20
0
Survival (%)
DMSO
Gö 6976
Ro 32-0432
4
6
8
10
Time (h)
B
100
80
60
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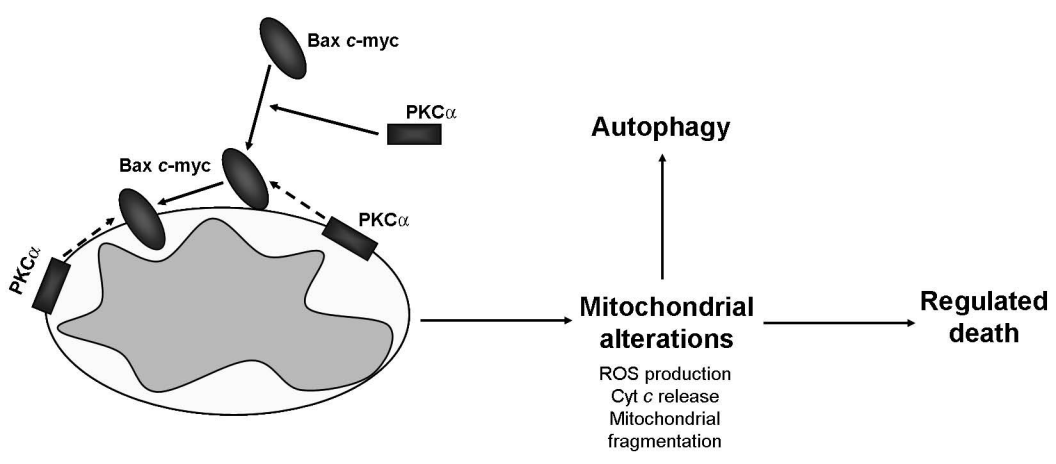
% Survival
PKCα
Bax c-myc
PKCα K368R
Bax c-myc
6
8
Time (h)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PKCα
Bax c-myc
PKCα
PKCα
Autophagy
Mitochondrial alterations
ROS production
Cyt c release
Mitochondrial fragmentation
Regulated death