Impact of pH on Bax α conformation, oligomerisation and mitochondrial integration

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Abstract The change in the conformation of Bax at the onset of apoptosis is a determinant for the execution of this cell death programme. However, very few models can account for this modification and the factors involved in this process remain elusive. We have analysed the modifications in the conformation induced by a variation in pH using a cell-free assay. We show that a moderate basic or acidic pH can induce apoptotic-like changes in the conformation of Bax, such as the exposure of the N-terminal or the BH3 domain. These changes in the conformation are associated with the binding of Bax to mitochondria and an enhanced Bax homo- and oligomerisation. Our results suggest that variations in the pH, in a range consistent with that often observed during apoptosis, are sufficient to trigger Bax translocation to mitochondria and the subsequent release of apoptogenic factors from this organelle.

Keywords: Bax conformation; Apoptosis; Oligomerisation; Mitochondria; pH

1. Introduction

Members of the Bcl-2 family are the final regulators of apoptosis, one of the major cell death programmes, induced by a variety of physiological situations and death inducers [1]. The pro-apoptotic protein Bax α (Bax) is normally contained in an inactive conformation in healthy cells and its activation during the early phases of apoptosis is associated with changes in the conformation [2]. These changes involve not only the unblocking of the addressing signal of Bax and the insertion into the mitochondrial outer membrane, but also the subsequent oligomerisation step [3]. However, the structural changes involved in the transition from a monomeric inactive state to an oligomeric membrane-embedded conformation are not completely understood [2]. It is likely that this important step is under the control of numerous physiological factors, possibly of different natures [4]. It has been proposed that proteins like Bid, a BH3-only protein, play an important role in the translocation of Bax from the cytosol to mitochondria by inducing a change in the conformation and possibly chaperoning Bax en route to the mitochondria [5].

Several in vitro studies have shown that variations in the pH are often observed during the early steps of Bax activation and it has been proposed that a rise or a fall in pH was required for the activation of Bax [6–9]. However, MNR analysis of Bax has shown that its 3-D structure is not sensitive to changes in pH [10]; ruling out that the pH could be, at least directly, involved in the physiological apoptotic process. Nonetheless, a variation in pH has been shown to enhance the association of Bax with mitochondria in a cell free assay and in vitro [7,11]. In this study, we have investigated the effect of the pH on the different steps of activation of Bax using a cell free assay viz, the exposure of its N-terminal and BH3 domain, the induction of its association with mitochondria and the formation of homo- or hetero-oligomers.

2. Materials and methods

2.1. Reagents

Unless specified otherwise, all reagents used in this study were from Sigma (St. Louis, MO, USA). Monoclonal Bax antibodies 2D2, AF820 and 6A7 were from R&D Systems (Lille, France) and the polyclonal anti-Bax BH3 TL41 was obtained as described previously [12]. Anti-cyt *c* antibody was from Pharmingen (#54854).

2.2. Association of Bax with mitochondria in the cell free assay

Mitochondria were prepared from normal rat liver and the cell-free association of Bax with the mitochondria was performed as described previously [11]. Briefly, ^{35}S-Met-proteins were synthesised from cDNAs using the TNT coupled transcription/translation system from Promega (Lyon, France). The post-translational insertion of in vitro translated (IVT) proteins into mitochondrial membranes and the alkaline treatment of mitochondrial-bound proteins were performed in a standard import buffer as described previously [11]. ^{35}S-Met-proteins bound to the mitochondria were recovered in the pellet after centrifugation for 10 min at 4 °C at 8000 × *g*. ^{35}S-Met-Bax associated with isolated mitochondria was analysed in a 12% SDS–PAGE and scanned with a Phosphorimager (Molecular Dynamics, France). The liberation of cyt *c* induced by Bax was determined by anti-cyt *c* immunoblots of the supernatant of the above cell free assay. The amount of protein present in the gels was subsequently quantified using the IP-Lab gel programme (Signal Analytics, Vienna, VA, USA).

2.3. Immunoprecipitation of Bax

For immunoprecipitation (IP) experiments, ^{35}S-Met-Bax mutants were incubated in the presence of Bax antibodies: 2D2, 6A7, Ab-4 or AF820 for 1 h at 4 °C. The antibody/protein complexes were then

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Abbreviations: Bax, Bax α; cyt *c*, holocytochrome *c*; IP, immunoprecipitation; ^{35}S-Met-Bax, in vitro translated Bax; RFP, red fluorescence protein

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precipitated with protein G– or protein A–agarose followed by centrifugation and washes according to the manufacturer's instructions (Zymed, USA). Briefly, IVT Bax was incubated at 4 °C for 10 min with 10 μl non-immune IgG serum and 50 μl protein G–agarose. The mixture was centrifuged for 10 min at 10000×*g* and the supernatant transferred to a new tube, to which 4 μg of Bax antibodies was added together with the secondary antibodies (Sigma) coupled to either protein G– or protein A–agarose. After an additional incubation with agitation of 1 h at 4 °C, the mixture was centrifuged at 3000 × *g* for 2 min, then washed several times in PBS before a final centrifugation. Samples were kept in SDS–PAGE loading buffer at −80 °C for no more than 2 weeks before SDS–PAGE and autoradiography.

2.4. Modification of pH and trypsin treatment of Bax

The pH was, adjusted as described in [13,14] with NaOH or HCl. Trypsin treatment of Bax was performed as described in [11], except that, when Bax was incubated at various pH, the pH was adjusted to 7.4 before proteolysis.

2.5. Detection of the pH-induced homo- or oligomerisation of Bax

Low-binding Ultracel-YM membrane from Millipore (France) with cut-offs of 30 kDa (MicroCon YM 30) or 50 kDa (MicroCon YM 50) was used to isolate IVT Bax monomers (20 kDa) or dimers (40 kDa), respectively, as recommended by the manufacturer. IVT Bax incubated at various pH were first added to microCon YM 30 and the retained fractions were reloaded on the microCon YM 50. The presence of IVT Bax in the eluted fractions from both columns was verified by SDS–PAGE and autoradiography.

2.6. Oligomerisation of Bax with Bcl-2 and Bcl-xl

IVT Bax was incubated at different pH. The association of Bax with Bcl-2 and Bcl-xl was performed using purified His-tagged full-length Bcl-2 or Bcl-xl as described by Cartron et al. [15,16], except that IVT Bax was incubated at various pH prior to the addition of the His-tagged anti-apoptotic proteins and binding to Ni–NTA agarose. Bound Bax/Bcl-2 or Bax/Bcl-xl complexes was eluted with 250 mM imidazole [15,16] and the amount of Bax determined as described in Section 2.2.

2.7. Cross-linking

The cross linker DSS (*N*-hydroxysuccinimide ester) was added to IVT Bax at a final concentration of 5 mM in 1 ml. After incubation for 30 min at room temperature, the cross-linker was quenched by the addition of 1 M Tris–HCl (pH 7.5). Bax oligomerisation was analysed by 12% SDS–PAGE gels, followed by Phosphorimager analysis.

2.8. Confocal studies of Bax changes of conformation

Apoptosis was induced in human glioblastoma cell lines by 20 μM staurosporine (STS), a broad range kinase inhibitor, as described in [17]. Laser confocal microscopy was performed as described earlier [17] using antibodies raised against different Bax epitopes.

3. Results

3.1. Change in pH triggers Bax association with mitochondria

IVT Bax was incubated in the presence of rabbit reticulocyte at different pH (from pH 4 to 10) for 30 min at 37 °C. At the end of the incubation, the pH was adjusted to 7.4 with NaOH or HCl prior to incubation with mitochondria (see Section 2.2). As previously reported [11,16,18], at pH 7.4 Bax binds poorly to mitochondria in the cell free assay and this is still true when the pH was increased to pH 7.8 or decreased to pH 6.8 (Fig. 1A). In all cases, none of the mitochondrial-bound Bax α was membrane embedded as judged by its resistance to an alkaline treatment. Conversely, at pH 8.2 or 6.0 Bax binding to mitochondria was significantly increased and most of the protein was membrane embedded, indicating that Bax

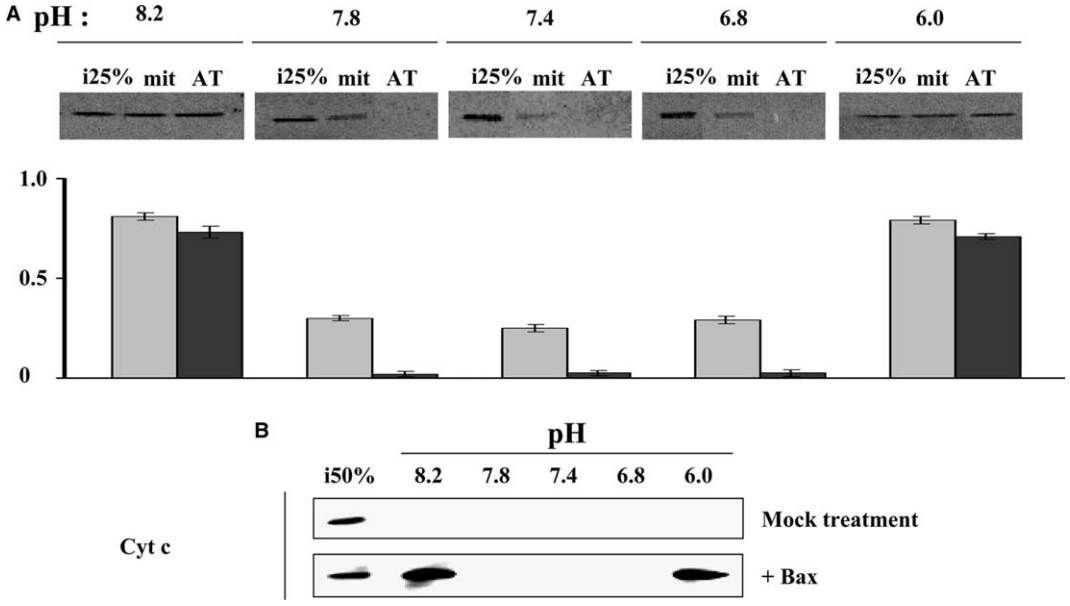

Fig. 1. Influence of the pH on the interaction of Bax α with mitochondria in a cell free assay. (A) ^{35}S-Met-Bax was incubated at different pH prior to neutralisation at pH 7.4 and the addition of 50 μg rat liver mitochondria in standard import buffer as described in Section 2. At the end of the incubation (1 h at 37 °C), the mitochondria were recovered by centrifugation and the presence of IVT Bax directly analysed on SDS–PAGE followed by fluorography. In some cases before centrifugation, mitochondria were treated with 100 mM Na_2CO_3 (pH 11) (alkaline treatment: AT) before analyses. i25% = 25% of the IVT Bax added. Histograms show the amount of IVT Bax present in the gels. Data shown are means ± S.D. obtained from at least four independent experiments. (B) Bax-induced release of cyt *c* was analysed by immunoblotting the supernatant from mitochondria incubated above. Only membrane embedded (i.e., AT-resistant) IVT Bax caused the release of cyt *c* for mitochondria. Experiments shown are representatives of at least three independent experiments. i50% = 50% of cyt *c* present in 50 μg rat liver mitochondria.

was fully inserted into the mitochondria membrane (Fig. 1A). In the latter case and contrary to that observed with a Bax pre-incubated at pH 6.8–7.8, cyt *c* was released from mitochondria, demonstrating that a pretreatment at pH 8.2 or at pH 6.0 of Bax induced a functional change in the conformation (Fig. 1B). As a control, we used a plasmid encoding for a cytosolic red fluorescence protein (RFP) incubated at various pH. As shown in Fig. 1B, under these conditions, no liberation of cyt *c* was observed.

3.2. pH triggers two types of changes in the conformation of Bax

The conformational changes induced by a variation in the pH were analysed by IP of IVT Bax using different antibodies (Fig. 2A). The 2D2 antibody is directed against an N-terminal peptide (amino acids 3–16) and cross-reacts with Bax regardless of its inactive/cytosolic or active/mitochondria conformation [19,20]. The anti-conformational antibody 6A7 directed against amino acids 12–24 [19,20] and the antibodies AF820, TL-41 and Ab-4, which are, respectively, directed against the N-terminal amino acids 12–34, 57–69 (i.e., the BH3 domain) and 98–117 (i.e., the α5α6 helices). As illustrated in Fig. 2B, a variation in the pH triggered selective IP with the different antibodies: exposure of the BH3 domain was induced in Bax pretreated at a pH superior to 7.6 and inferior to 7.0, while exposure of the N-terminus was observed only when ^{35}S-Met-Bax was incubated at a pH superior to 8.2 or inferior to 6.0. A change in the sensitivity of a protein towards mild proteolysis is an indicator of structural modification. As shown in Fig. 2C, in the range of pH from 7.8 to 6.8, ^{35}S-Met-Bax was highly sensitive to trypsin proteolysis, while at a pH superior to 8.2 or inferior to 6, it became resistant to trypsin. Of note, similar results were obtained using proteinase K (data not shown). Taken together (Fig. 2D), these results suggest that two major changes in the conformation are induced by pH. The first occurred at a pH between 6.8 and 6 or between 7.6 and 8, in which the BH3 domain of Bax became accessible to antibody binding. The second occurred at a pH inferior to 6 or superior to 8, as observed by its resistance to a trypsin treatment and by IP using antibodies directed against the N-terminus and the helix α5. Thus, from these data one can conclude that the first step in the change in the conformation of

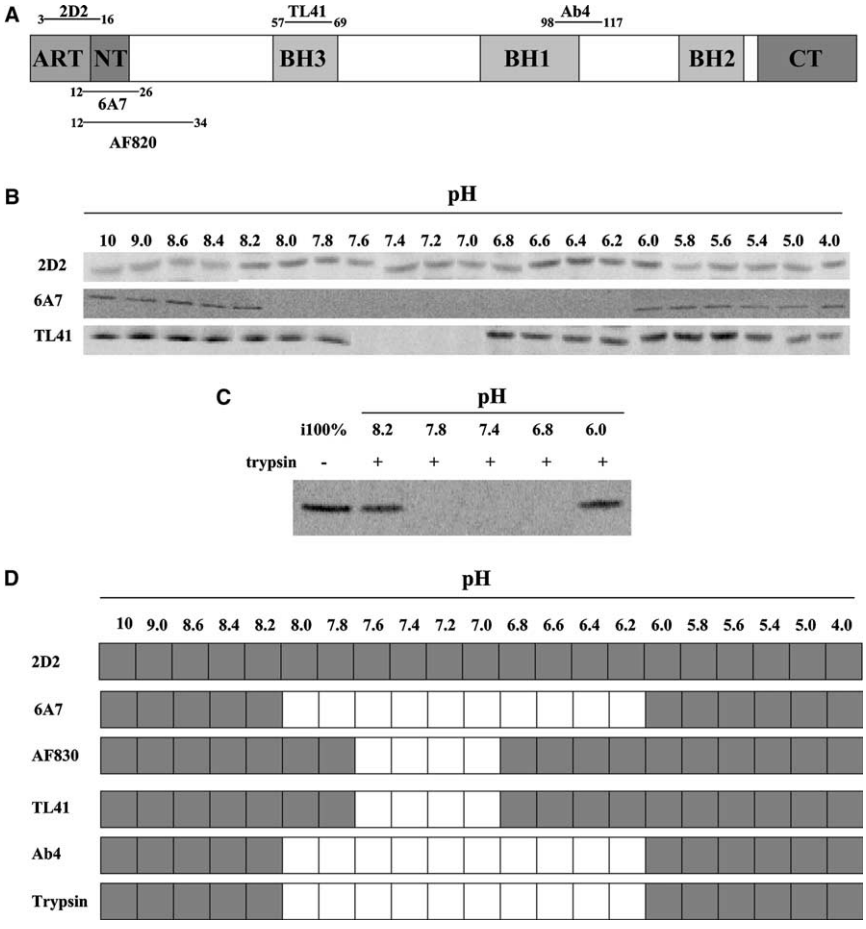

Fig. 2. Influence of pH on epitopes accessibility and sensitivity to a trypsin treatment. (A) Schematic representation of the epitopes recognised by the different antibodies used in this study as well as the different Bax domains. Localisation of the epitopes recognised by the different antibodies as indicated by the manufacturers (see Section 2) and BH domains are illustrated as described by Suzuki et al. [10]. (B) IVT Bax was incubated at different pH before IP with different antibodies, followed by SDS–PAGE/fluorography analysis as described in Section 2. (C) IVT Bax was incubated with trypsin (1 mg/ml for 10 min at 4 °C), then proteolysis was stopped by the addition of 10 mg/ml soybean trypsin inhibitor and immediately denatured in SDS–PAGE buffer as described in [13]. Fluorographs illustrated are representatives of at least three independent experiments. (D) Schematic representation of the results obtained with epitopes accessibility (i.e., IP) and trypsin resistance of IVT Bax incubated at different pH from 10 to 4. Shaded squares indicate a positive IP or resistance to trypsin treatment, while the clear squares indicate no IP or sensitivity to trypsin treatment.

Bax involves the BH3 domain and the second step the exposition of the N-terminal, which contains its addressing signal [17] and the helix α1, part of the putative pore-forming domain that has been shown to be the membrane insertion domain of Bax α [21].

To address the physiological significance of these findings, we used laser confocal microscopy to study the time course of the appearance of Bax epitopes in human glioma cells treated with 2 μM STS. As illustrated in Fig. 3, the 2D2 antibody recognises both the mitochondrial and the cytosolic fractions of Bax. We observed that most of the cytosolic Bax was translocated to mitochondria after 5 h and that this subcellular localisation was accompanied by the exposure of the 6A7 epitopes [19]. In agreement with our results with pH, the exposure of the BH3 epitope (i.e., labelling with TL41) occurred before that of the N-terminus (i.e., labelling with 6A7) (Fig. 3).

3.3. Shift to low or high pH triggers Bax homo- and hetero-oligomerisation

Xie et al. [8] showed that an acidic pH promoted the dimerisation among Bcl-2 family proteins. We investigated Bax homo-dimerisation using a simple experimental setting in which Bax monomer was separated from the dimers by simple filtration using MicroCon filters with different cut-offs (see Section 2.4 and Fig. 4A). As shown in gel filtration experiments, at pH 7.4, Bax was present in the eluant of MicroCon YM-30 and was not detectable in the fraction retained by MicroCon YM-30 (data not shown). This result suggested that it was present solely in a molecular weight inferior to 30 kDa and, thus, most probably as a monomer (Fig. 4B). Conversely, when Bax was preincubated at pH 8.2, it was retained in the MicroCon YM-30 but eluted from the MicroCon YM-50, suggesting that this pH induced dimerisation. The specificity of this technique was assessed using a Bax mutant (Bax K64E) that is unable to dimerise (Cartron et al., in preparation). At pH 7.4 or 8.2, Bax K64E was detected only in the eluted fractions of the MicroCon YM-30 (Fig. 4B), confirming that this construct did not undergo homo-dimerisation.

To confirm the pH induced-dimerisation, IVT Bax or Bax K64E were cross-linked using DSS and the cross-linked products were analyzed on SDS–PAGE and autoradiography as described in Section 2. As shown in Fig. 4C, the molecular weights of the cross-linked products were consistent with our previous experiment and show a BH3-dependent pH-induced dimerisation.

Next, we investigated the effect of the pH on the formation of hetero-dimers between Bax and Bcl-xl or Bcl-2. Since Bax can form hetero-dimers with Bcl-2 and Bcl-xl at pH 7.4, we did not use the filtration on MicroCon, as it is essentially a qualitative method. Instead, we analysed the pH-induced dimerisation of Bax with His-tagged Bcl-2 or Bcl-xl using a method previously reported [16], as it allowed a quantitative evaluation of the association of Bax with anti-apoptotic proteins [15]. As shown in Fig. 4D, increasing the pH from 7.4 to 8.2 or decreasing it from 7.4 to 6.0 similarly enhanced the binding of Bax to both Bcl-2 and Bcl-xl. In this case, since the increase of hetero-oligomerisation was observed at pH 7.8, we concluded that the conformational constraint required for the binding of Bax with Bcl-2 or Bcl-xl was different from that required for its homodimerisation. Of note, Bax K64E did not oligomerise with either Bcl-2 or Bcl-xl under these conditions.

4. Discussion

The alkalisation of the intracellular compartment has been shown to induce Bax translocation in vitro in different types of cells and by different apoptotic stimuli [6,7,9]. From these results, a model has been elaborated wherein the intramolecular interactions of the N-terminus and the C-terminus of Bax, which secured its "inactive" state, could be altered by modification of the pH [7]. This modification can be either an acidification or an alkalisation as shown by Khaled et al. [7] that both positively and negatively charged amino acids could be involved in the pH-induced conformational change. We used different antibodies which recognise different epitopes in Bax

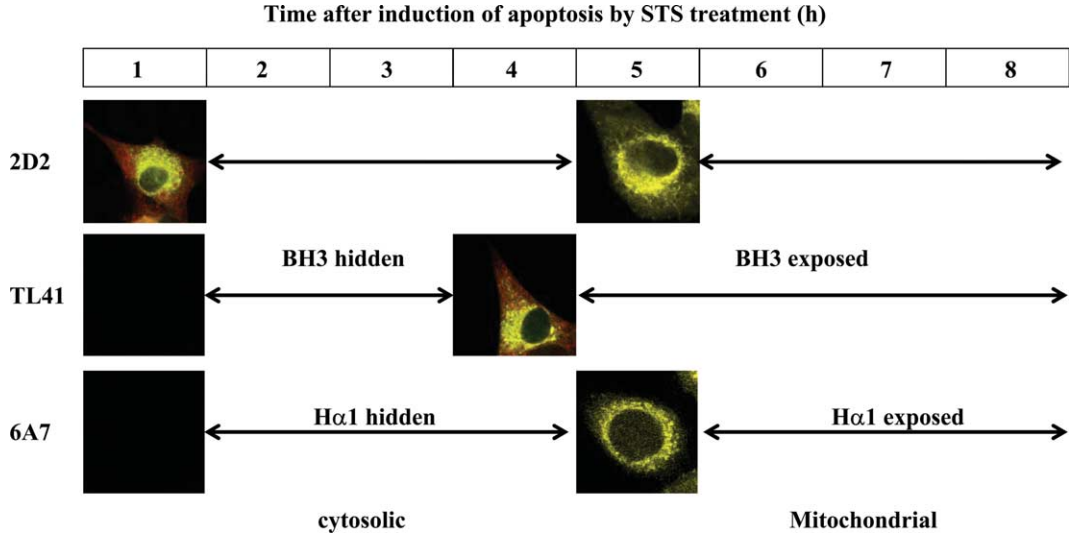

Fig. 3. Confocal analysis of the change of conformation of Bax. Human glioma cells [23] were treated with 20 μM STS, then Bax was labelled with the anti-Bax antibodies 6A7, 2D2 and TL41 every hour over a period of time of 8 h. As illustrated in this figure, the labelling with the anti-2D2 antibody was observed throughout this period of time, while labelling with TL41 and 6A7 was observed, respectively, after 4 and 5 h. The illustration is representative of at least five different experiments.

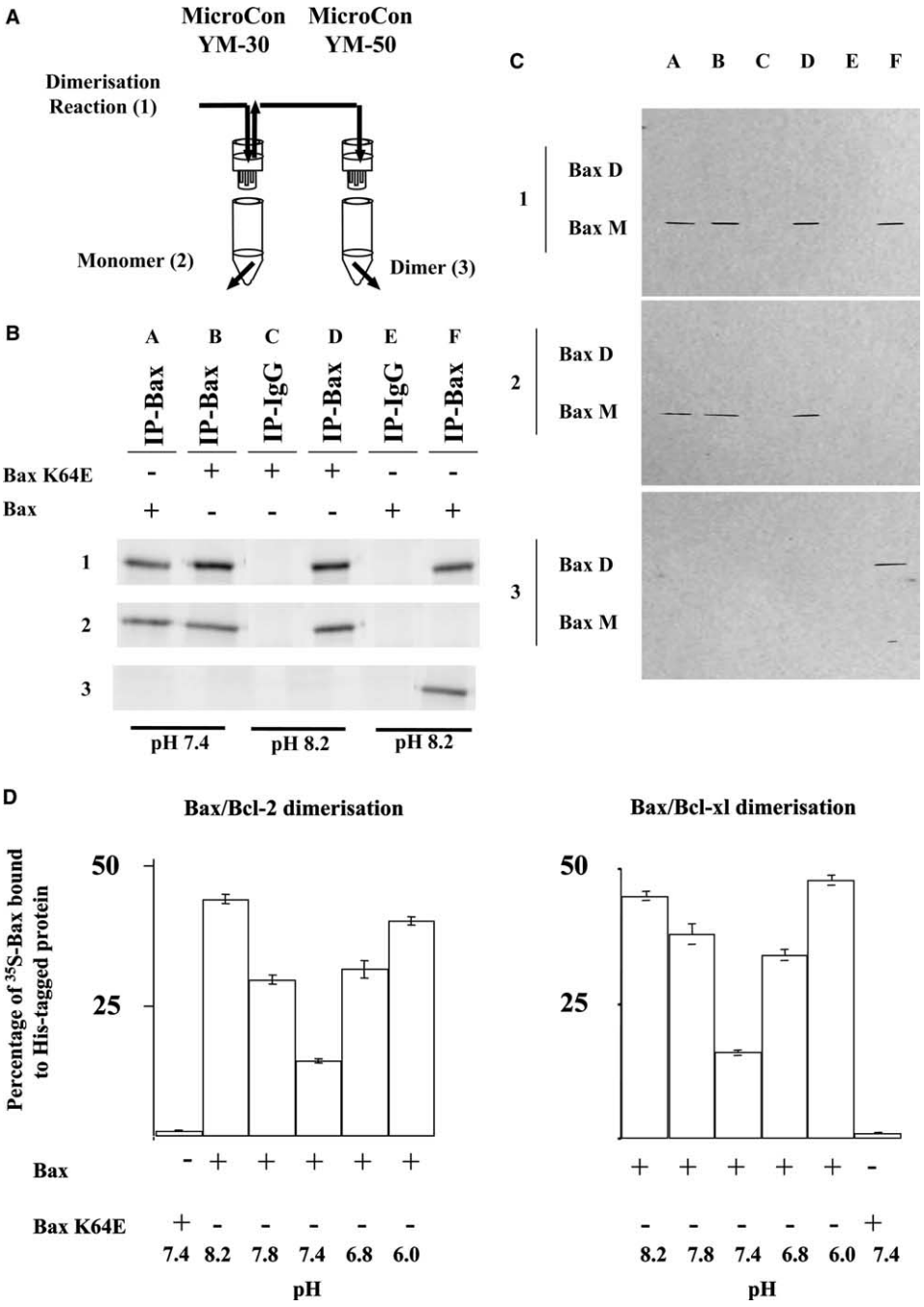

Fig. 4. pH-induced homo- and hetero-oligomerisation of Bax. (A) Schematic representation of the experimental settings used to analyse homo-dimerisation of Bax. IVT Bax or Bax K64E [1] was filtered through MicroCon YM as described in Section 2. According to the cut-off, only monomers can be recovered with MicroCon YM-30 [2]. Samples not filtered through MicroCon YM-30 (thus, with a molecular weight superior to 30 kDa) were reloaded on a MicroCon YM-50 and the presence of Bax analysed in filtrated fraction [3]. The fraction of Bax not filtered through MicroCon YM-30 but filtrated on MicroCon YM-50 is thus likely to be a dimer (i.e., molecular weight $\geqslant$ 30 kDa and $\leqslant$50 kDa). Larger oligomers or aggregates, however, were not observed in the fraction retained on MicroCon YM 50 (data not shown). Thus, the absence of Bax in the second filtration (i.e., MicroCon YM-50) means that all the Bax was present as a monomer. (B) IVT Bax or Bax K64E [1], incubated at two pH (i.e., pH 7.4 and 8.2), filtered through MicroCon YM-30 [2] and MicroCon YM-50 [3]. Since ^{35}S-Met-Bax (Bax) or Bax K64E was eluted in a large volume (about 500 μl), Bax was immunoprecipitated, as described in Section 2, with the 2D2 antibody (IP-Bax) or with non-immune IgG, as a control for non-specific IP (IP-IgG). Autoradiography shown is representative of 3 independent experiments. (C) IVT Bax and Bax K64E were cross-linked at pH 7.4 or 8.2 and their molecular weight analysed on SDS–PAGE and autoradiography as described in Section 2. M = monomer, MW 21 kDa; D = dimer, MW 42 kDa. (D) The amount of IVT Bax oρ B$\alpha\xi$ K64E associated with His-tagged Bcl-xl or Bcl-2 was determined as described in Section 2. Data are means ± S.D., calculated from at least three independent experiments.

to determine the sequence of events involved in the change of conformation induced by pH (Fig. 2A). We show that the 2D2 antibody immunoprecipitated Bax at all pH studied, while the 6A7 antibody recognises this protein only at pH superior to 8.6 or inferior to 6.8 (Fig. 2B). These different sensitivities can be related to a change of conformation involving a sequence

encompassing amino-acids 12-24 (i.e., the 6A7 epitope) which is hidden at neutral pH as originally proposed [20]. Our results are completely in agreement with the above model as we show that a transient incubation of Bax at acidic or basic pH is sufficient to trigger irreversible changes in the conformation consistent with that observed during apoptosis as the monoclonal antibody 2D2 recognises Bax both in resting and in apoptotic cells, while the monoclonal antibody 6A7 and the polyclonal antibody TL41 recognised Bax only in apoptotic cells (Fig. 3). However, MNR studies have shown that shifting the pH from 6 to 8 did not significantly affect the structure and the oligomerisation state of Bax [10], contrary to experimental data reported here and elsewhere [8]. The reason for this discrepancy is unclear but it might reflect the fact that the conformation of Bax analysed by Suzuki et al. [10] is only partial. Indeed, the structure of the first 12 amino acids of the protein, which play an essential role in apoptosis [16,18] could not be resolved by Suzuki et al. [10]. It is also possible that the purification procedure after expression in the bacteria induced a conformation that could not be altered any further.

It has been reported that the exposure of internal region of Bak, another pro apoptotic multidomain protein of the BCl-2 family, was a secondary step and was preceded by the exposure of the NT [22]. Here, we show that changes of conformation of Bax follow a different route as STS induced apoptosis promotes first the exposure of the BH3 domain of Bax and as a secondary step that of Hα1 (Fig. 3). These differences could be related to the fact that Bax change of conformation accompanies a change of subcellular localisation contrary to Bak which is a mitochondria protein.

The exact impact of the pH on Bax function during in vivo apoptosis still remains to be established. However, we show in this work that simple shifts in the pH, compatible with that observed in a cellular model, can reproduce in a cell free assay some of the essential steps involved in Bax activation during apoptosis: viz. exposition of the BH3 domain, the N-terminal conformational epitope 6A7 and that of the helix α1. Quite remarkably, the variations in the pH, which promote Bax addressing to mitochondria in a cell free assay (Fig. 1), also provoke its homo- or hetero-oligomerisation (Fig. 4), suggesting that these events could occur in parallel in vivo but do not necessarily involve mitochondrial elements. In conclusion, studies on the conformational changes in Bax induced by the pH could allow a better understanding of the energy required and/or the domain(s) involved in Bax translocation and integration within the mitochondria.

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