Comparative Biophysical Characterization of p53 with the Pro-apoptotic BAK and the Anti-apoptotic BCL-xL

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The p53 transcription-independent apoptosis in mitochondria, mediated by its interaction with the pro-apoptotic and the anti-apoptotic members of the Bcl2 family of proteins, has been described in vivo, especially in radiosensitive tissues. We have characterized the interaction of p53 with both the pro-apoptotic Bak and the anti-apoptotic Bcl-xL proteins, comparing their affinity and their interaction surfaces, using biophysical techniques such as fluorescence anisotropy, analytical ultracentrifugation, and NMR. We have shown that both proteins interact with only the p53 core domain and not with its N- and C-terminal regions. Further, p53 has a higher affinity for Bcl-xL than for Bak, which is consistent with the previously described sequential binding of Bcl-xL and Bak by p53. Interestingly, although the interaction with both proteins is electrostatic in character, they have different binding sites. Using NMR spectroscopy, we have determined that Bcl-xL interacts with the DNA binding site of p53, but Bak does not interact with this site. A new potential interaction surface for Bak is proposed.

p53 is a transcription factor that acts as a tumor suppressor by inducing growth arrest or apoptosis in response to a variety of cellular stresses among other activities (1–4). These responses protect the cell against uncontrolled proliferation, and more than 50% of human cancers have mutations in the gene coding for p53 (5). p53 is a homotetramer, with each monomer consisting of several independent domains: the N-terminal transactivation domain (residues 1–63); the proline-rich regulatory domain (residues 64–92); the DNA binding core domain (residues 94–312); the tetramerization domain (residues 324–355); and the C-terminal domain (residues 360–393). In response to multiple death stimuli, p53 rapidly translocates to mitochondria, promoting apoptosis via a transcriptionally independent pathway (6). This p53 transcription-independent apoptosis precedes p53 target gene activation (7) and is mediated by the direct interaction of p53 with at least five of the members of the Bcl2 family, the anti-apoptotic members Bcl2 and Bcl-xL (8), and the pro-apoptotic Bax (9), Bak (10), and Bad (11).

The Bcl2 family members regulate the apoptosis mediated by mitochondria. They are defined by the presence of at least one of the four Bcl2 homology domains (BH1, BH2, BH3, or BH4). Despite their similar structures, they are divided into two subfamilies of opposite functions: the anti-apoptotic subfamily (including Bcl2, Bcl-xL, Bclw, and Mcl1) and the pro-apoptotic subfamily (including Bax, Bak, Bok/Mtd, and the BH3-only proteins Bik, Puma, and Nox) (12). The exact mechanism used by the Bcl2 family to control apoptosis in mitochondria is not completely understood, but it is known that the pro-apoptotic members are active only when they oligomerize. Further, it has been proposed that oligomerization of pro-apoptotic members is inhibited upon the binding of anti-apoptotic members (13).

 Interestingly, p53 is able to bind both pro-apoptotic and anti-apoptotic members of the Bcl2 family. The binding of p53 to Bcl2 and Bcl-xL inhibits their anti-apoptotic function. Although the exact mechanism of this inhibition is not known, it has been proposed that the interaction of p53 could prevent or disrupt the complex between the anti-apoptotic and the pro-apoptotic proteins (10, 14, 15). Further, the interaction between p53 and the pro-apoptotic proteins Bax or Bak activates them (9, 10), inducing conformational changes that promote the oligomerization that is required to exert their pro-apoptotic function. Several reports support the importance of the p53 transcription-independent apoptosis pathway in vivo. Thus, p53 translocates to mitochondria in radiosensitive tissues of an irradiated mouse (7), targeting of p53 to mitochondria suppresses growth of lymphomas in vivo (16), and the inhibition of the binding of p53 to Bcl-xL protects mice from radiation (17). Detailed knowledge of this pathway, including the characterization of the interaction between p53 and the Bcl2 family proteins is, therefore, very important for future anti-tumor therapies or radioprotection of normal tissues in radiotherapy. Although there are several biochemical studies of these interactions (14), there are few biophysical characterizations of the binding of the core domain of p53 to the anti-apoptotic Bcl-xL (15) and Bcl2 (18), and there are no biophysical data regarding its binding to the pro-apoptotic proteins. In this work, we have biophysically characterized the binding of p53 to Bak and Bcl-xL and compared them. Our results show that Bcl-xL interacts with higher affinity than Bak, but both proteins bind only the core domain and not the C- and N-terminal regions of p53. Further, using competition binding experiments and NMR spectroscopy we have shown that the interaction surface of Bak differs from the site described for Bcl-xL, and a new potential binding site is proposed. The physiological significance of these findings is discussed.

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EXPERIMENTAL PROCEDURES

**Plasmid Construction**—BakΔC3 (residues 1–190) and Bcl-xLΔC (residues 1–211) coding sequences, without the C-terminal transmembrane domain, were amplified from a cDNA library (Clontech, multiple tissue cDNA panels) from heart and brain, respectively. Amplified PCR products were cloned into the pGEMT vector system (Promega). The expression vectors for both proteins were made by inserting the coding sequence into the pRSETHisLipoTEV vector (19) using an overlapping PCR strategy (20). Using this strategy, the proteins cleaved from the fusion proteins contained only an additional N-terminal glycine.

**Protein Expression and Purification**—Escherichia coli C41 containing the expression plasmids for Bcl-xLΔC and BakΔC were grown at 37 and 25 °C, respectively, to log phase, when 0.5 mM isopropyl 1-thio-D-galactopyranoside (IPTG) was added. Bcl-xLΔC was expressed for 4 h at 37 °C, and Bak was expressed for 15 h at 20 °C. The proteins were purified using nickel affinity chromatography (Qiagen). The lysates, resuspended in binding buffer (50 mM potassium phosphate, pH 8, 300 mM NaCl, 1 mM mercaptoethanol, and 10 mM imidazole), were loaded onto the equilibrated column and eluted with the elution buffer (50 mM potassium phosphate, pH 8, 300 mM NaCl, 1 mM mercaptoethanol, and 250 mM imidazole). The fusion protein was digested with TEV protease and reapplied to the nickel affinity column to remove the His-lipo-TEV tag. The proteins were recovered in the flow through, then diluted 10 times in 25 mM Tris (pH 8.0), 50 mM NaCl, 1 mM DTT, 2 mM EDTA, and loaded onto a source 30Q anion exchange column (Amersham Biosciences) equilibrated in the same buffer. The proteins were eluted using a NaCl gradient (0.05–1 M). The final step of the purification was gel filtration chromatography using a Superdex 75 HiLoad 26/60 (Amersham Biosciences) equilibrated in 50 mM Tris (pH 8.0), 200 mM NaCl, 1 mM DTT, and 2 mM EDTA. For Bak purification, all the buffers contained 10% glycerol. The p53 core domain (p53c) (residues 94–312), p53 full-length quadruple mutant (p53 flQM), and the N-terminal region of p53 (p53N) (residues 1–93) were purified as described (19, 21).

**Fluorescein Labeling of Proteins**—Proteins were labeled using 5′ and 6′ carboxyfluorescein succimidyl ester (Molecular Probes) as described (20). To separate the non-incorporated fluorescein, the mixture was loaded onto an analytical GF column Superdex 75 10/300 GL (Amersham Biosciences). This chromatographic step also provided confirmation that the labeling process had not affected the protein conformation. The yield of labeled protein varied between 20 and 40% of the total protein. The final product consisted of a mixture of both labeled and unlabeled species.

**Fluorescence Anisotropy**—The experiments were performed at 10 °C with a PerkinElmer LS 55 spectrofluorimeter equipped with a Hamilton Microlab M dispenser, using an excitation wavelength of 480 nm and an emission wavelength of 530 nm. A concentrated solution of protein was titrated into 1 ml containing fluorescein-labeled proteins. Titrations were carried out at three ionic strengths (50, 100, and 150 mM). The 50 mM ionic strength buffer (I, 50 mM) was 20 mM sodium phosphate, pH 7.5, and 1 mM DTT. For the higher ionic strength buffers (I, 100 and 150 mM), NaCl was added to a final concentration of 48 and 97 mM, respectively. The program Kaleidagraph™ (Synergy Software, Reading, PA) was used to calculate the dissociation constants by fitting the polarization data to one binding site plus linear drift equation (22).

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 r = r_0 + (r_1\text{Titrant})/(K_d + \text{Titrant}) + c\text{Titrant}
\]  

(\text{Eq. 1})

where \( r \) is the experimentally determined anisotropy at each point of the titration, \( r_0 \) is the initial anisotropy, \( r_1 \) is the difference in anisotropy between unbound and bound to one titrant molecule, \( K_d \) is the dissociation constant, \( \text{Titrant} \) is the molar concentration of titrant (M), and \( c \) is the constant of the linear drift.

In the competition binding assays, the double-stranded GADD45 response element sequence, assembled from the high pressure liquid chromatography-purified oligonucleotides 5′-CAGAACATGTCTAAGCATGCTGGG-3′ and 5′-CAGAACATGTCTAAGCATGCTGGG-3′, was titrated into a mixture of 150 mM fluorescein-labeled protein (FL-Bcl-xL ΔC or FL-Bak ΔC) and 27 μM p53C. For the Bcl-xL ΔC and Bak ΔC competition experiments, Bcl-xL ΔC was titrated in a mixture of 150 mM FL-Bak ΔC and 20 μM p53C.

**Analytical Ultracentrifugation**—Equilibrium sedimentation experiments were carried out at 10 °C with a Beckman Optima XL-I ultracentrifuge in a 50 Ti rotor at 18,000 rpm. The total concentration of the fluorescein-labeled proteins FL-Bcl-xL and FL-Bak was 15 μM and of the unlabeled p53C was 100 μM at the three different ionic strengths. Absorbance at 492 nm (fluorescein absorbance maximum) and interference data were measured. The absorbance data were used to calculate the concentrations of free-labeled protein and its complex, by fitting the data to a double-exponential model corresponding to two species in equilibrium. As the concentration of p53C is much higher than the concentration of labeled protein, the contribution of the latter and the complex to the interference data can be discarded. Thus, the interference data were used to calculate the total p53C concentration (approximately equal to free concentration) by fitting the data to a single exponential model.

### Footnotes

3 The abbreviations used are: BakΔC, C-terminal deleted Bak (residues 1–190); Bcl-xLΔC, C-terminal deleted Bcl-xLΔC (residues 1–211); FL, fluorescein; FL-BakΔC, fluorescein-labeled BakΔC; FL-Bcl-xLΔC, fluorescein-labeled Bcl-xLΔC; Np53, the N-terminal region of p53 (residues 1–93); p53C, the p53 core domain (residues 94–312); p53CT, the C-terminal region of p53 (residues 293–392); p53 flQM, p53 full-length quadruple; DTT, dithiothreitol; AUC, analytical ultracentrifugation; TEV, tobacco etch virus.

4 Dr. Gorka Basañez, personal communication.
corresponding to one species present. Data were processed and analyzed using ULTRASPIN software. The dissociation constants were calculated using a single model binding equation.

$$K_d = \frac{([\text{free FL-protein}] \cdot [\text{free p53C}])}{[\text{complex}]} \quad (\text{Eq. 2})$$

Controls of fluorescein protein and p53C were carried out in parallel to check the oligomerization state of the proteins and their integrity at the end of the centrifugation experiment.

NMR—$^{1}H, ~^{15}N$ heteronuclear single quantum correlation NMR spectra were acquired on a Bruker DRX-600 spectrometer equipped with CryoProbe™ and single axis gradients, at 298 K ($I$, 100 MM) in buffer (20 mm sodium phosphate, pH 7.5, 48 mM NaCl, and 1 mM DTT). The concentrations of the proteins were 100 μM $^{15}N$-p53C and 300 μM Bcl-xLΔC or BakΔC. The effects of binding were analyzed using the assignments of p53C (23). The data were processed in Felix98 (MSI Biosystems), and figures were generated using NMRView (24).

RESULTS

Interaction of Bcl-xL and Bak with p53C—The interaction of p53 with Bcl-xLΔC (8) and Bcl2ΔC (18) has been described. The binding surface for these proteins overlaps with the DNA binding surface of p53; therefore, an electrostatic binding has been suggested (15). Further, biochemical experiments suggest that Bak also binds to this domain (10). We analyzed the binding of BakΔC and Bcl-xLΔC to the core domain of p53 (p53C) at different ionic strengths (50, 100, and 150 mM) to compare both interactions and their electrostatic nature (Fig. 1). Two biophysical techniques were used to test the binding using fluorescein-labeled Bcl-xLΔC and BakΔC: fluorescence anisotropy (Fig. 1A) and analytical ultracentrifugation (AUC) (Fig. 1B) (Table 1). The anisotropy measurements revealed an electrostatic interaction between p53C and both BakΔC and Bcl-xLΔC since affinity decreased with increasing ionic strength. Further, the interaction of p53C with BakΔC appeared weaker than that observed for Bcl-xLΔC. The interactions were too weak for measurement $K_d$ except for Bcl-xLΔC at 50 mM ionic strength ($K_d$ 16 μM; Table 1), where the binding reached near saturation and could be fitted to a one-binding site model plus linear drift. We also analyzed these interactions by AUC, which was a powerful technique for measuring $K_d$ for weaker binding (Fig. 1B) (Table 1). The sedimentation curves of FL-Bcl-xLΔC and FL-BakΔC without p53C could be fitted to a single model of a monomeric protein of 24 and 20 kDa, respectively, but the protein mixture sedimentation profiles fitted to a bi-exponential

![FIGURE 1. Binding of p53C to Bcl-xLΔC and BakΔC at different ionic strengths. A, fluorescent anisotropy binding curves at 50, 100, and 150 mM ionic strength. 150 nM fluorescein-labeled protein was titrated with unlabeled p53C. Solid line in the binding curve for Bcl-xLΔC (I, 50 mM) corresponds to the fitting to a single site binding model plus linear drift. B, equilibrium sedimentation analysis for the binding at ionic strengths 50 and 150 mM. Solid lines correspond to fit to one or two species present. Lower panels represent the residuals for the fittings.]

| TABLE 1 | Dissociation constants of Bcl-xLΔC and BakΔC from p53C at different ionic strengths ($I$) |
|----------|---------------------------------|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| $I$       | Bcl-xLΔC                        | BakΔC                          | $K_d$ (μM)     | $n$             | $K_d$ (μM)     | $n$             |
| NaN       | NaN                             | NaN                            | NaN            | NaN             | NaN            | NaN             |
| 50        | $16 \pm 6^b$                    | $162 \pm 37$                  | 10             | 12              | $162 \pm 37$  | 10              |
| 100       | $79 \pm 26$                     | $290 \pm 85$                  | 14             | 10              | $290 \pm 85$  | 14              |
| 150       | $151 \pm 8$                     | $500 \pm 100$                 | 9              | 5               | $500 \pm 100$  | 9               |

*a* Number of experiments used to calculate the average.

*b* Calculated from anisotropy experiments because binding was too tight to be measured by AUC. All others calculated from AUC experiments.
model of a mixture of two species of 24 and 49 kDa for mixtures of p53C and FL-Bcl-x\textsubscript{L}\textsubscript{ΔC} and of 20 and 45 kDa for mixtures of p53C and FL-Bak\textsubscript{ΔC}. For Bcl-x\textsubscript{L}\textsubscript{ΔC} at 50 mM ionic strength, binding was too tight to be measured by AUC; all the Bcl-x\textsubscript{L}\textsubscript{ΔC} was bound to p53C, and the sedimentation curve could be fitted to only a single species of 49 kDa, consistent with the $K_d$ calculated by anisotropy. These experiments also confirmed weak and electrostatic binding for both proteins as well as the higher affinity for p53C of Bcl-x\textsubscript{L}\textsubscript{ΔC} than of Bak\textsubscript{ΔC}, as can be observed from the calculated $K_d$s summarized in Table 1.

Binding to the N- and C-Terminal Domains of p53—Although it is clear that Bcl-x\textsubscript{L} and Bak interact with the DNA binding domain of p53 (10, 15) and confirmed here, the role of the N-terminal region (residues 1–94) of p53 (Np53) in the interaction with and in induction of p53 transcription-independent apoptosis is controversial. A deletion mutant of p53 without the proline-rich domain (residues 62–91) is unable to activate Bax (9), a pro-apoptotic protein analogue of Bak. Further, NMR binding experiments imply a direct interaction between Bcl-x\textsubscript{L} and Np53 (25). In contrast, it is claimed that the N-terminal domain of p53 is not able to bind Bcl-x\textsubscript{L} and Bak (8, 10). To investigate whether either protein can interact with this region of p53, we checked the binding of Bcl-x\textsubscript{L}\textsubscript{ΔC} and Bak\textsubscript{ΔC} to fluorescein-labeled Np53 (residues 1–93) by fluorescence anisotropy (Fig. 2A). Bcl-x\textsubscript{L}\textsubscript{ΔC} and Bak\textsubscript{ΔC} did not bind to Np53 under our conditions, although Np53 interacted with the p53 interaction domain of MDM2 (residues 2–125). This interaction has been already described (26) and was used as a control.

In contrast to the controversy about the role of the N-terminal domain of p53 in transcription-independent apoptosis, it is commonly accepted that the C-terminal domain is not important for the mitochondrial function of p53 (6). No biophysical in vitro experiments have been reported to check the interaction of this domain with the Bcl-2 family proteins. Here, the interaction of the fluorescein-labeled C-terminal domain of p53 (p53CT, residues 293–392) with Bcl-x\textsubscript{L}\textsubscript{ΔC} and Bak\textsubscript{ΔC} was thus checked by fluorescence anisotropy (Fig. 2B). The binding of S100B, a protein that binds p53CT (20), was used as a positive binding control. In this case, as for the N-terminal domain of p53, there was no binding of Bcl-x\textsubscript{L}\textsubscript{ΔC} and Bak\textsubscript{ΔC} to p53CT.

The interaction of both the proteins with full-length Tp53, a thermodynamically stable quadrupole mutant of p53 (27), was also tested (data not shown). Unfortunately, the interaction was too weak to be able to calculate the $K_d$ by fluorescence anisotropy, as the concentration of p53 was limited to <30 μM under the experimental conditions at higher ionic strengths, and p53 aggregated at the lower ionic strengths where binding was tighter and more readily measured. Our results show that the interaction was weak, with a $K_d$ higher than 50 μM, which might indicate that there was no cooperative effect between core domains to bind Bak and Bcl-x\textsubscript{L}.

**Determination of the Binding Surface of the p53C-BakΔC Complex**—NMR data has previously shown (15) that p53C and Bcl-x\textsubscript{L}\textsubscript{ΔC} interact via the DNA binding region of p53, suggesting a electrostatic nature of the binding, which is confirmed by our binding experiments at different ionic strengths. Because the binding to Bak\textsubscript{ΔC} is also ionic strength-dependent and therefore electrostatic, the question arises whether it also binds to the same binding site. To check this possibility, we performed fluorescence anisotropy competition assays for binding of p53C between unlabeled DNA (GADD45 response element) and fluorescein-labeled FL-Bak\textsubscript{ΔC} or FL-Bcl-x\textsubscript{L}\textsubscript{ΔC} (Fig. 3). The $K_d$ for the interaction between p53C and GADD45 is 14 μM at 210 mM ionic strength (28), but at lower salt concentration the affinity is higher, with $K_d$ in the mM range (29). As can be seen in Fig. 3, the DNA was able to disrupt completely the pre-formed complex with both proteins at the three ionic strengths, as revealed by the reversal of the increment in anisotropy obtained on addition of 27 μM p53C to both fluorescein-labeled proteins. DNA displacing the proteins at three ionic strengths demonstrated that the binding surface for each was the same at three ionic strengths, i.e. the higher affinity at low salt concentration was not because of nonspecific binding but due to a higher electrostatic potential between the binding surfaces.

An important observation made from these experiments was that a higher DNA concentration was needed to disrupt the p53-Bak\textsubscript{ΔC} complex than for the p53-Bcl-x\textsubscript{L}\textsubscript{ΔC} complex, despite the higher affinity of the latter. This suggests that the DNA binding site of p53 and the Bak\textsubscript{ΔC} binding surface do not overlap completely as in the case for Bcl-x\textsubscript{L}\textsubscript{ΔC}. To test this possibility, the $^1$H, $^{15}$N heteronuclear single quantum correlation spectra for both complexes were recorded to compare the binding site in p53C for both proteins. The experiments were performed in 100 mM ionic strength buffer at a 1:3 molar ratio, p53C: Bak\textsubscript{ΔC} or Bcl-x\textsubscript{L}\textsubscript{ΔC}, respectively. Our results obtained with Bcl-x\textsubscript{L}\textsubscript{ΔC} are consistent with the previously published
data (15) (Fig. 4A), confirming that the Bcl-xLΔC binding site overlaps the DNA binding surface of p53 (Fig. 5A). However, the binding of BakΔC did not produce the same signal perturbations in the p53C spectrum (Fig. 4A), confirming the suggestion that BakΔC did not bind to the DNA binding site. The residues of p53C perturbed upon binding of BakΔC were localized between the amino acids 202 and 208, as well as Val-173, which was severely line broadened because of this interaction. This region was not perturbed by the presence of Bcl-xLΔC, as can be seen for Tyr-205 in Fig. 3B. Because of the low affinity of p53C with BakΔC, all the perturbations were small or led only to an exchange broadening of peaks, as would be expected from a low affinity site with an exchange rate of hundreds of μs. However, the fact that all the affected residues were located on the same protein surface (Fig. 5A) supports the idea that this might be a binding site for Bak. These residues are situated on the surface between monomers that is formed when two core domains bind DNA (30). This fact may explain the competition observed between DNA and Bak for binding p53C because when two p53Cs interact with DNA and form the protein-protein interactions that stabilize the core dimer bound to DNA, this would present steric hindrance for the binding of Bak. Thus, the competition would be indirect, which would explain the higher DNA concentration needed to break the complex with BakΔC than the complex with Bcl-xLΔC.

To check further the possibility of different binding sites for both proteins, we performed competition experiments between Bcl-xLΔC and BakΔC at 50 mM ionic strength buffer (Fig. 5B). Under these conditions, the affinity of p53C for Bcl-xLΔC is 10 times higher than for BakΔC. Thus, if the binding sites of both proteins overlap, Bcl-xLΔC could easily disrupt the complex between BakΔC and p53. Bcl-xLΔC was not able to disrupt the complex between BakΔC and p53, and it can be concluded that the binding sites are independent.

**DISCUSSION**

Here, we have shown, using several techniques, that Bcl-xL and Bak interact with the p53 core domain. Both interactions are highly electrostatic in nature, being quite weak at physiological ionic strength. A low affinity for the interaction between p53 and the Bcl2 family proteins has been described previously by an NMR study of the complex between Bcl-xL and p53C (15) and by the surface plasmon resonance study of the interaction of Bcl2 and p53C (18). However, Tomita et al. described a two binding site interaction, with a high affinity interaction ($K_D$, 535 nM) followed by a weaker one ($K_D$, 16.9 μM). But we did not see this high affinity binding for Bcl-xLΔC and BakΔC using fluorescence anisotropy and AUC. Chipuk et al. (38), using Biacore, also showed a nanomolar affinity for the interaction between recombinant Bcl-xL and mammalian-expressed cytoplasmic protein.
Interaction of p53 with Bcl2 Family Members

As it is known that p53, once in the mitochondria, can bind Bcl-xL and Bak, inhibiting one and activating the other, it would be interesting to know if there is any preference for one of them, providing a sequential mechanism of action, or if the affinity for both is the same, and the pathway involves binding randomly to either of them. Previously, a sequential mechanism has been proposed: the first effect of p53 is the inhibition of Bcl-xL followed by a second step of activation of Bak/Bax (38). Our quantitative data are consistent with this sequential mechanism. We have shown that p53 has higher affinity for Bcl-xL; therefore under the conditions of equal concentrations of pro-apoptotic and anti-apoptotic proteins or of higher concentration of the later, p53 would preferentially bind Bcl-xL. The interaction with Bak would occur as a second step, if there is an excess of p53 molecules or, as presented by Chipuk et al. (38), the BH3-only proteins release p53 from Bcl-xL. Further, the higher affinity for the anti-apoptotic proteins can be correlated with the different functions that these interactions promote. Thus, an inhibitory interaction must be tighter than an activating interaction, for which a transient interaction might be enough. Is this low affinity enough to prevent binding between the anti- and the pro-apoptotic members? Although the exact mechanism of the inhibition of apoptosis by Bcl-xL and Bcl2 is unknown, one of the hypotheses is that in unstressed cells Bak and Bax are not capable of oligomerizing. Binding with the activator BH3-only proteins produces a conformational change that activates their oligomerization (39). The anti-apoptotic proteins Bcl2 and Bcl-xL only bind to the “activated” form of Bak and Bax, inhibiting them before their oligomerization. Following this hypothesis and the sequential binding of p53, once p53 activates Bak, Bcl-xL is already inactivated by p53.

Our results have demonstrated that Bcl-xL and Bak do not interact with the N- and C-terminal domains of p53, in agreement with other studies (6, 8, 10). Further, the low affinity of full-length Tp53 for both proteins suggests that there is no strong co-operativity between monomers of p53 in binding these proteins. In addition, the low affinity of full-length Tp53

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**FIGURE 5.** Bcl-xLΔC and BakΔC bind in different sites on p53C. A, location of disappearing or changing chemical shifts induced by the binding of Bcl-xLΔC and BakΔC to a dimer of p53C bound to DNA (Protein Data Bank code 2ADY). Residues are colored according to their electrostatic nature: acidic in red, basic in blue, polar in yellow, and apolar in gray. B, fluorescence anisotropy competition assays between Bcl-xLΔC and BakΔC for binding p53C. Bcl-xLΔC was tritiated into a solution of 150 nM fluorescein-labeled BakΔC (●) or into 150 nM fluorescein-labeled BakΔC and 20 μM p53C (●). The increase of anisotropy induced by the binding of p53C to 150 nM fluorescein-labeled BakΔC is shown in the left panel. The buffer used was 20 mM sodium phosphate, pH 7.5, 1 mM DTT (I 50 mM).

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full-length p53. In this case, it could happen that the post-translational modifications, present in mammalian-purified p53, might increase the affinity. But bacterially expressed p53 can oligomerize Bak in isolated mitochondria and can bind Bcl-xL with the same efficiency as protein expressed in mammalian cells (8). Further, the immobilization of proteins to a solid phase support can theoretically affect rate constants by 10-fold compared with solution measurements (31).

Weak protein-protein interactions are a common phenomenon in the cell. For example, the $K_d$ for the interaction of the small GTPase Ras with its activator p120GAP is 17 μM in buffer without salt and 32 μM when 20 mM NaCl is added (32). The interaction between lymphocyte cell surface proteins is of very low affinity, which is favorable for reversible cell-cell adhesion processes (33, 34). Further, vesicular transport is controlled by an intricate network of specific but weak protein-protein interactions (35). These interactions, as with p53-Bcl2 family binding, occur in places where the concentration of the proteins is locally enriched, i.e. the implicated proteins are transported to the same place. Thus, for the activity of p53 in mitochondria, this low affinity would allow regulation, avoiding the induction of apoptosis in cells that should not be apoptotic. In this way, transcription-independent apoptosis would occur only when death stimulus is produced and mono-ubiquitinated. p53 (36) is specifically transported to mitochondria, increasing the local concentration of p53. A weak transient interaction (named “hit and run” process) has also been proposed for the binding of p53 and Bax (37) because a physical binding between these two proteins cannot be detected by co-immunoprecipitation (8, 9), despite the fact that direct activation of Bax by p53 has been shown in a lipid system free of proteins except p53 and Bax.
for both proteins suggests that there is no strong co-operativity between monomers of p53 in binding these proteins. This lack of co-operativity is consistent with the finding that a monomeric truncation mutant of p53 without the oligomerization domain is able to produce the same levels of transcription-independent apoptosis as wild-type p53 (6). Each subunit in tetrameric p53 thus appears to act independently. The quaternary structure of p53 appears, indeed, to be a very loosely coupled system in which each core domain is relatively independent (40).

In an in vitro membrane-free system, Bak is not able to bind its BH3-only protein activator BID (BH3 Interacting Domain death agonist) (41) and its inhibitor Bcl-xL (Fig. 5B). The structural explanation is that the hydrophobic groove formed by the BH1, BH2, and BH3 domains, used by the Bcl-2 family for mutual interaction, is occluded (41). p53 can bind Bak is a fact that suggests that p53 does not bind to this groove. p53 binds Bcl-xL, at an acidic site in the BH4 domain that does not overlap with this groove. Further, Moldoveanu et al. (41) have shown that Zn\(^{2+}\) has an inhibitory effect on Bak. We assayed the binding of p53C and Bak in the presence of Zn\(^{2+}\) (data not shown) but observed no difference compared with the binding in its absence. Thus, p53 may be able to activate Bak even in the presence of this inhibitor.

The DNA binding interface of p53 is basic in character and binds the acidic surfaces of most of the proteins that interact with the core domain of p53, such as HIT-1, 53BP2, 53BP1, and Bcl-xL. Bak and Bak, however, do not have a very acidic protein surface (41, 42) nor do they have a BH4 domain, the interacting domain of Bcl-xL. This makes the binding of these proteins to the DNA binding site unlikely. Our NMR results confirm that Bak does not bind to this site, opening the possibility of a new binding site for Bak. This interface, although electrostatic in character, possesses both acidic and basic residues (Fig. 5A), having more in common with the overall surface of Bak. The fact that p53 has different binding sites for the anti- and the pro-apoptotic members of the Bcl2 family may explain the apoptotic activity in mitochondria of the contact mutant R273H, which is able to release cytochrome c but is not able to bind Bcl-xL (8) and Bcl2 (18). In this mutant, the surface interacting with Bak is unaffected by the mutation, therefore it may bind Bak and produce apoptosis.

It is important to note that, although in the in vitro competition experiments between Bcl-xL and Bak both proteins were able to bind the core domain simultaneously, this may not reflect the reality of an in vivo situation, in which Bcl2 proteins are anchored in the membrane, and p53 is tetrameric. Spatial and steric restrictions might not permit the interaction with both at once.

In conclusion, the work presented here provides important biophysical data about the interaction of Bcl-xL and Bak with p53 that support a mechanism of sequential interaction of p53 in mitochondria, in which p53 interacts preferentially with the anti-apoptotic members of the Bcl2 family, inhibiting them and secondly with the pro-apoptotic, activating them. Further, we demonstrated different binding sites for Bcl-xL and Bak and have suggested the location of the binding site for Bak. These facts will be of special importance for future anti-tumor or radioprotection therapies.

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