

## Domain-dependent Photodamage to Bcl-2

A MEMBRANE ANCHORAGE REGION IS NEEDED TO FORM THE TARGET OF PHTHALOCYANINE PHOTSENSITIZATION\*

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**Photodynamic therapy using the photosensitizer Pc 4 and red light photochemically destroys the antiapoptotic protein Bcl-2 and induces apoptosis. To characterize the requirements for photodamage, we transiently transfected epitope-tagged Bcl-2 deletion mutants into DU-145 cells. Using confocal microscopy and Western blots, wild-type Bcl-2 and mutants with deletions near the N terminus were found in mitochondria, endoplasmic reticulum, and nuclear membranes and were photodamaged. A mutant missing the C terminus, including the transmembrane domain, spread diffusely in cells and was not photodamaged. Bcl-2 missing  $\alpha$ -helices 5/6 was also not photodamaged. Bcl-2 missing only one of those  $\alpha$ -helices, with or without substitutions of the singlet oxygen-targeted amino acids, behaved like wild-type Bcl-2 with respect to localization and photodamage. Using green fluorescent protein (GFP)-tagged Bcl-2 or mutants in live cells, no change in either the localization or the intensity of GFP fluorescence was observed in response to Pc 4 photodynamic therapy. Western blot analysis of either GFP- or Xpress-tagged Bcl-2 revealed that the photodynamic therapy-induced disappearance of the Bcl-2 band was accompanied by the appearance of bands indicative of heavily cross-linked Bcl-2 protein. Therefore, the  $\alpha_5/\alpha_6$  region of Bcl-2 is required for photodamage and cross-linking, and domain-dependent photodamage to Bcl-2 offers a unique mechanism for activation of apoptosis.**

Photodynamic therapy (PDT)<sup>1</sup> is a novel treatment for cancer and certain noncancerous conditions that are generally characterized by overgrowth of unwanted or abnormal cells (1, 2). The procedure requires exposure of cells or tissues to a photosensitizing drug followed by irradiation with visible light of the appropriate wavelength, usually in the red or near infrared region and compatible with the absorption spectrum of the drug. Since the first modern clinical trial of PDT by Dougherty *et al.* (3) was reported in 1978, PDT with the pho-

tosensitizer Photofrin<sup>®</sup> has been applied to many solid tumors and is approved by the United States Food and Drug Administration for the treatment of advanced esophageal, early lung, and late lung cancers. In order to enhance the efficacy of PDT and extend its applications, a variety of second generation photosensitizers, such as the silicon phthalocyanine Pc 4, are now being assessed for their efficacy in cancer therapy, and it is important to elucidate their mechanisms of action in PDT.

The photosensitizers for PDT are primarily porphyrins or porphyrin-related macrocycles, such as phthalocyanines, benzoporphyrins, purpurins, and pyropheophorbides (4, 5). Because of their hydrophobic aromatic ring structures, they localize to one or more cellular membranes. We have reported that Pc 4 preferentially binds to the mitochondrial membrane, the endoplasmic reticulum, and Golgi complexes in cancer cells (6, 7). Upon photoactivation, mitochondrial reactive oxygen species are produced, and these are critical in initiating mitochondrial inner membrane permeabilization, which leads to mitochondrial swelling, cytochrome *c* release to the cytosol, and apoptotic death (7). PDT produces singlet oxygen and other reactive oxygen species in the membranes and causes photooxidative damage to proteins and lipids that reside within a few nm of the photosensitizer binding sites (8, 9). One important target of Pc 4-PDT (10) as well as PDT with certain other photosensitizers (11) is the antiapoptotic protein Bcl-2.

The Bcl-2 family consists of proteins that either promote or inhibit apoptosis. Proapoptotic members include Bax and Bak, whereas antiapoptotic members include Bcl-2 and Bcl-x<sub>L</sub>. They share 1–4  $\alpha$ -helical Bcl-2 homology (BH) domains, whose subtle differences in primary sequence and spatial arrangements may determine the pro- and antiapoptotic functions. The antiapoptotic proteins, like Bcl-2, possess four BH domains (BH1–BH4) (12). The C-terminal regions of many members of the Bcl-2 family consist of a stretch of hydrophobic amino acids that serves to anchor the proteins to intracellular membranes, specifically the outer mitochondrial membrane, the endoplasmic reticulum, and the nuclear envelope (13). Bcl-2 contains a transmembrane domain of 19 amino acids at the C terminus, and it has been reported that deletion of the C-terminal 22 amino acids of Bcl-2 abrogates cellular membrane attachment (14, 15).

Structural analysis of Bcl-x<sub>L</sub> reveals its resemblance to the bacterial pore-forming toxins, colicin and diphtheria toxin, especially in the  $\alpha$ -helical region that mediates membrane insertion of the toxins (16, 17). This region corresponds to the BH1 domain and part of the BH2 domain of Bcl-2 and Bcl-x<sub>L</sub>, and is called the  $\alpha_5/\alpha_6$  region. This region is thought to be inserted into the membrane and form ion channels (18–20). It has been reported that mutations within the  $\alpha_5/\alpha_6$  region of Bcl-2 and

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<sup>1</sup> The abbreviations used are: PDT, photodynamic therapy; Pc 4, phthalocyanine 4; Pc 4-PDT, PDT with Pc 4; BH, Bcl-2 homology; ER, endoplasmic reticulum; PBS, phosphate-buffered saline; TM, transmembrane.

Bcl-x<sub>L</sub> abrogate the antiapoptotic activity and block the heterodimerization with other members of the Bcl-2 family, such as Bax or Bak, which are death-promoting proteins. The region between the BH1 and BH2 domains forms part of a hydrophobic cleft and may be the site of interaction with Bax or Bak (16, 17).

The role of Bcl-2 in the apoptotic response caused by PDT has remained controversial. He *et al.* (21) first reported that overexpressing human Bcl-2 made Chinese hamster ovary cells more resistant to apoptosis and to loss of clonogenicity upon exposure to Pc 4-PDT. In contrast, Kim *et al.* (11) found that overexpression of Bcl-2 in MCF-10A cells caused the up-regulation of Bax and enhanced cell killing and apoptosis by PDT with ALPc. Recently, Srivastava *et al.* reported that antisense-Bcl-2 sensitized A431 cells to Pc 4-PDT (22). As in the study by Kim *et al.* (11), the cells overexpressing Bcl-2 were more sensitive to induction of apoptosis than were the parental cells, because of up-regulation of Bax; however, no studies were done to determine whether those cells were sensitized to overall cell death.

We have reported that Pc 4-PDT photodamages Bcl-2, as detected by Western blot analysis as the immediate loss of the native 26-kDa protein (10). Bcl-2 photodamage was selective, in that several other mitochondrial proteins were not affected. Kessel and Castelli (23) found that PDT with three different photosensitizers destroyed Bcl-2 but not Bax. In contrast, a recent report from Antieghem *et al.* (24) showed that PDT with the nonporphyrin photosensitizer, hypericin, does not cause Bcl-2 destruction but produces a G<sub>2</sub>/M delay, during which Bcl-2 becomes transiently phosphorylated. Although it is now clear that Bcl-2 is one target of PDT with Pc 4 and some other photosensitizers, the mechanism of the photodestruction of Bcl-2 has not been defined. In order to elucidate the structural features that determine photosensitivity, we constructed Bcl-2 mutants by site-directed mutagenesis and examined the association between their subcellular localization and their sensitivity to photodestruction by Pc 4-PDT. Furthermore, we compared the responses of wild-type and mutant Bcl-2 to those of the proapoptotic proteins, Bak and Bax. Our photobiological analysis of the Bcl-2 family members suggests a relationship between membrane localization and photosensitivity.

#### EXPERIMENTAL PROCEDURES

**Cell Culture**—Human prostate cancer DU-145 cells were grown in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum. Human breast cancer MCF-7 cells transfected with human procaspase-3 cDNA (MCF-7c3 cells) were cultured in RPMI 1640 medium containing 10% fetal bovine serum (25). Human breast epithelial MDA-MB-468 cells were cultured in Dulbecco's modified Eagle's medium. All cultures were maintained in a humidified atmosphere at 37 °C with 5% CO<sub>2</sub>.

**Plasmid Construction**—An expression vector housing full-length human Bcl-2 cDNA inserted at the *EcoRI* site, pUC19-Bcl-2, was kindly provided by Dr. C. W. Distelhorst (Case Western Reserve University) (26). Bcl-2 cDNA was digested with *EcoRI* and cloned into the mammalian expression vector pcDNA4/HisMax (Invitrogen), which encodes an N-terminal peptide containing a polyhistidine metal-binding tag and the Xpress epitope. It was also cloned into the N-terminal green fluorescent protein (GFP) mammalian expression vector pEGFP-C3 (Clontech, Palo Alto, CA). To generate the Bcl-2 mutants, mutations were amplified from pUC19-Bcl-2 using the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA) (18, 19). The following mutagenic primers were used: for mutant a, Bcl-2 (Δ33–54) (5'-ATCTTCTCCTCCAGCCCGGGCAGACCCGGCTGCCCGGGC-3' (forward) and 5'-GCCGGGGCAGCCGGGGTCTGCCCGGGCTGGGAGGAGAGAT-3' (reverse)); for mutant b, Bcl-2 (Δ37–63) (5'-GTGGGATGCGGAGATGTGGGGCAGCCGGTCCGACGAGACC (forward) and 5'-GGTCTGGCGACCCGGTCCGCCATCTCCCGATCCAC-3' (reverse)); for mutant c, Bcl-2 (Δ10–125) (5'-CTGGGAGAACGGGGTACGACGCGGGGACGCTTTGCCAC-3' (forward) and 5'-GTGGCAAAGCGTCCCGCGCTGTACCCCGTCTCCAG-3' (reverse)); for mutant d,

Bcl-2 (Δ153–179) (5'-GGATTGTGGCCTTCTTTGAGTACCTGAACCGGCACCTGCAC-3' (forward) and 5'-GTGCAGGTGCCGGTTCAGGTA-CTCAAAGAAGGCCACAATCC-3' (reverse)); for mutant e, Bcl-2 (Δ153–168) (5'-GGATTGTGGCCTTCTTTGAGCTGGTGACAACATC-GCCC-3' (forward) and 5'-GGGCGATGTTGTCCACCAGCTCAAAGAA-GGCCACAATCC-3' (reverse)); for mutant f, Bcl-2 (Δ168–179) (5'-GC-GTCAACCGGGAGATGTCGTACCTGAACCGGCACCTGC-3' (forward) and 5'-GCAGGTGCCGGTTCAGGTACGACATCTCCCGGTTGACG-C-3' (reverse)); for mutant g, Bcl-2 (Δ210–239) (5'-GCCCCAGCATGGCCTCTGTGAAGTCAACATGCCTGCC-3' (forward) and 5'-GGCCAGGCATGTTGACTTCACAGAGCCGCATGCTGGGGC-3' (reverse)); for mutant h, Bcl-2 (Δ218–233) (5'-ATTCTCCTGGCTGTCTGGCCTATCTGAGCCACAAGTGA-3' (forward) and 5'-TCACTTGTGGCTC-AGATAGCCAGAGACAGCCAGGAGAAAT-3' (reverse)).

Additional Bcl-2 mutants were constructed to mutate specific amino acids. In order to generate mutant i, pUC19-Bcl-2 (Δ168–179) was amplified, and to generate mutant j, pUC19-Bcl-2 (Δ153–168) was amplified as a template for the QuikChange™ site-directed mutagenesis kit.

The following mutagenic primers were used: for mutant i, Bcl-2 (Δ168–179, F153L, M157I, C158V) (5'-CTTCTTTGAGCCGGTGGGGT-CATCGTGTGGAGAGCGTC-3' (forward) and 5'-GACGCTCTCCA-CAACGATGACCCACCGAGCTCAAAGAAG-3' (reverse)); for mutant j, Bcl-2 (Δ153–168, W176L, M177L) (5'-GTGGACAACATCGCCCTGT-TACTGACTGAGTACCTGAACC-3' (forward) and 5'-GGTTCAGG-TACTCAGTCAGTAAACAGGGCGCATGTTGTCCAC-3' (reverse)).

The reactions were carried out by 24 cycles of 30 s at 95 °C, 1 min at 55 °C, 12 min at 68 °C using *Pfu* polymerase (Stratagene, La Jolla, CA). The amplified products were digested with *DpnI* (Stratagene, La Jolla, CA), and the products were transfected into XL1-Blue supercompetent cells (Stratagene, La Jolla, CA). The amplified regions were confirmed by DNA sequence analysis and digested with *EcoRI* and then cloned to the mammalian expression vectors, pcDNA4/HisMax and pEGFP-C3.

**Photodynamic Therapy**—The phthalocyanine photosensitizer Pc 4, HOSiPcOSi(CH<sub>3</sub>)<sub>2</sub>(CH<sub>2</sub>)<sub>3</sub>N(CH<sub>3</sub>)<sub>2</sub>, was provided by Dr. M. E. Kenney (Department of Chemistry, Case Western Reserve University). Pc 4 was dissolved in dimethyl formamide to 0.5 mM. Cells were loaded with Pc 4 by the addition of an aliquot of the stock solution to the culture medium 16 h before irradiation. For all experiments, the light source was an EFOS light-emitting diode array (EFOS; Mississauga, Canada) delivering red light (λ<sub>max</sub> 670–675 nm; bandwidth at half-maximum 24 nm; fluence rate at the level of the cell monolayer, 6–7 milliwatts/cm<sup>2</sup>). The dose of PDT used in most of these experiments (200 nM Pc 4 plus 200 mJ/cm<sup>2</sup>) was demonstrated to produce 98 ± 4% killing of MCF-7c3 cells and 92 ± 2% killing of DU-145 cells, as determined by clonogenic assay.

**Western Blot Analysis**—Cells were harvested by centrifugation and washed twice with ice-cold phosphate-buffered saline (PBS). The cell pellets were incubated in a lysis buffer (50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 1% Triton X-100, 0.2% SDS, 0.5% deoxycholate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 100 mM NaF) on ice for 30 min and then sonicated. The protein content of the whole cell lysates was measured using the BCA protein assay reagent (Pierce). An aliquot (20 μg) of the whole cell lysate was separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were incubated with one of the following antibodies: mouse monoclonal anti-Xpress antibody (Invitrogen), rabbit polyclonal anti-human Bax antibody, rabbit polyclonal anti-human Bak antibody, mouse monoclonal anti-GFP antibody, and mouse monoclonal anti-actin antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at appropriate concentrations for 1 h. After rinsing with PBS containing 0.1% (v/v) Triton X-100, the membranes were incubated with anti-mouse or anti-rabbit immunoglobulin G conjugated to horseradish peroxidase for 1 h at room temperature. The membranes were washed and developed with Western blotting enhanced chemiluminescence detection reagents (Amersham Biosciences). Independent experiments were repeated at least three times.

**Fluorescence Immunocytochemistry**—DU-145 cells were grown on glass coverslips and then were transiently transfected with an expression vector (pcDNA/HisMax) encoding wild-type Bcl-2 or Bcl-2 mutants. Eight h after the transfection, Pc 4 was added to the cultures, which were further incubated for 16 h and then incubated with 100 nM MitoTracker Green (Molecular Probes, Inc., Eugene, OR) for 45 min at 37 °C. The coverslips were washed in PBS and fixed in 1% formaldehyde for 30 min. After rinsing twice with PBS, the fixed cells were incubated in IFA buffer (PBS containing 1% bovine serum albumin, 0.1% Tween 20) for 10 min and then IFA containing mouse anti-Xpress™ antibody (1:300 dilution; Invitrogen, CA) for 1 h at room



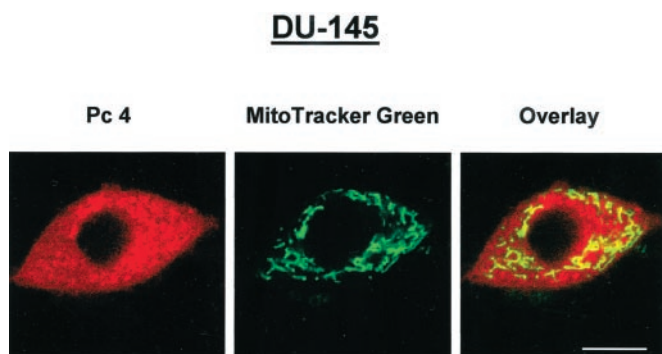


FIG. 1. **Pc 4 localizes to cytoplasmic membranes.** DU-145 cells were loaded with 200 nM Pc 4 for 16 h and 100 nM MitoTracker Green. The image of Pc 4 displayed a punctate pattern, but it did not completely co-localize with MitoTracker Green. Scale bar, 5  $\mu$ m.

temperature. After rinsing with IFA buffer to remove excess unbound antibody, the coverslips were incubated for at least 1 h at room temperature in IFA containing the second antibody, which was anti-mouse IgG conjugated to Texas Red (1:300 dilution; Vector Laboratories, Burlingame, CA). In all experiments, there was no detectable immunofluorescence signal above background in the absence of anti-Xpress<sup>TM</sup> antibody.

**Localization of Pc 4 in DU-145 Cells**—To investigate the subcellular sites of Pc 4 localization, DU-145 cells were plated on 35-mm coverslip dishes (MatTek Corp., Ashland, MA) and exposed to 200 nM Pc 4 at 37 °C for 16 h. To assess specific localization to mitochondria, cells were also incubated with 100 nM MitoTracker Green for 45 min at 37 °C.

**Confocal Microscopy**—All fluorescence images were acquired using a Zeiss LSM 510 inverted laser-scanning confocal fluorescence microscope in the Case Western Reserve University Ireland Comprehensive Cancer Center confocal microscopy facility. A  $\times$ 63 numerical aperture 1.4 oil immersion planapochromat objective was used. Confocal images of Pc 4 fluorescence were collected using 633-nm excitation light from a HeNe laser and a 650-nm long pass filter. Images of MitoTracker Green fluorescence were collected using 488-nm excitation light from an argon laser and a 500–550-nm band pass barrier filter. To investigate the localization of wild-type Bcl-2 and mutants, images of Texas Red were collected using 543-nm excitation light from a HeNe laser and a 560-nm long pass filter. For live cell fluorescence imaging of DU-145 cells transiently transfected with GFP-wild-type Bcl-2 or with GFP-Bcl-2 mutants, cells were plated on 35-mm glass bottom dishes (MatTek Corp., Ashland, MA) and transiently transfected (27). Eight h after the transfection, Pc 4 was added to the cultures, which were further incubated for 16 h and then incubated with 100 nM MitoTracker Red (Molecular Probes) for 45 min at 37 °C. Images of GFP fluorescence and MitoTracker Red fluorescence were collected using the same filter settings as for MitoTracker Green plus Texas Red, respectively (27). After taking these images, the cells were photoirradiated, and the same cells were imaged immediately and 1 h later.

## RESULTS

**Pc 4 Localizes to Mitochondria and Other Intracellular Organelles of DU-145 Cells**—In cells, PDT causes oxidative damage to target molecules that reside within a few nm of the sites of photoactivation of the photosensitizer (7, 8). We have previously shown that Pc 4 binds to mitochondria but also to various other organellar membranes, including the nuclear membrane and ER/Golgi membranes of murine lymphoma L5178Y-R cells (6) and human skin carcinoma A431 cells (7). Therefore, we first examined the localization of Pc 4 in DU-145 cells using confocal microscopy (Fig. 1). To assess whether Pc 4 binds to the mitochondria, DU-145 cells were co-loaded with MitoTracker Green, a mitochondrion-specific dye. The images of Pc 4 displayed a punctate pattern, but Pc 4 fluorescence only partially co-localized with MitoTracker Green fluorescence. These results suggest that in DU-145 cells Pc 4 localizes not only to mitochondria but also to the endoplasmic reticulum (ER), Golgi complexes, nuclear membrane, and possibly other intracellular organelles but to neither the plasma membrane

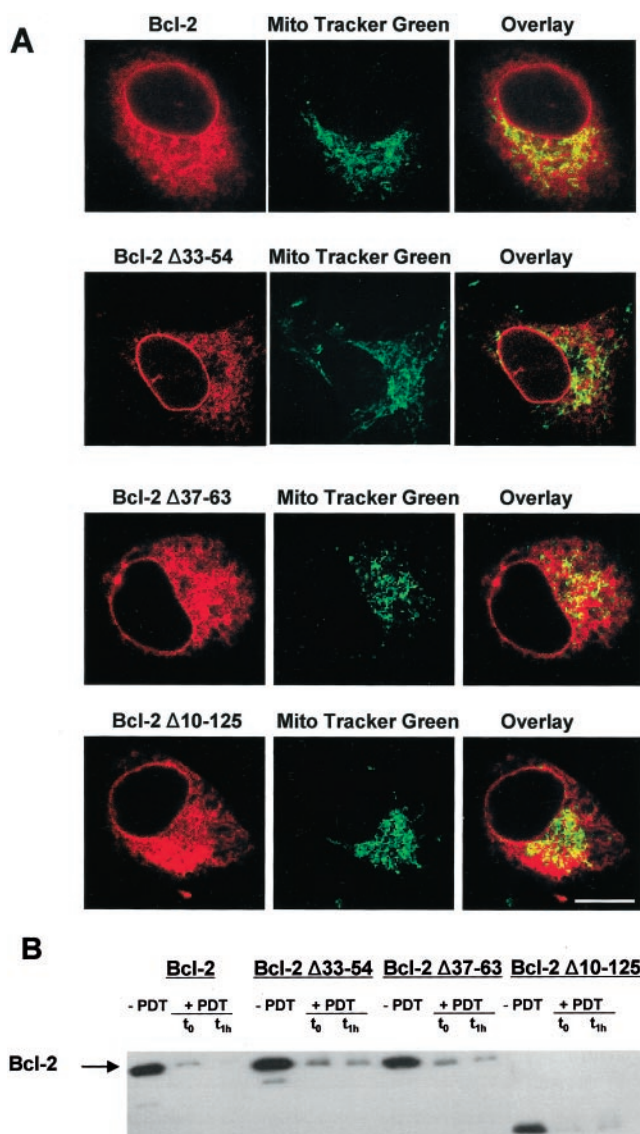


FIG. 2. **Design of Bcl-2 mutants.** A, the positions of the deleted regions within the human Bcl-2 protein are depicted. B, the mutants (a–h) are indicated with reference to the amino acids deleted from each.

nor the nucleus. The data are consistent with our earlier findings in other cell lines (6, 7).

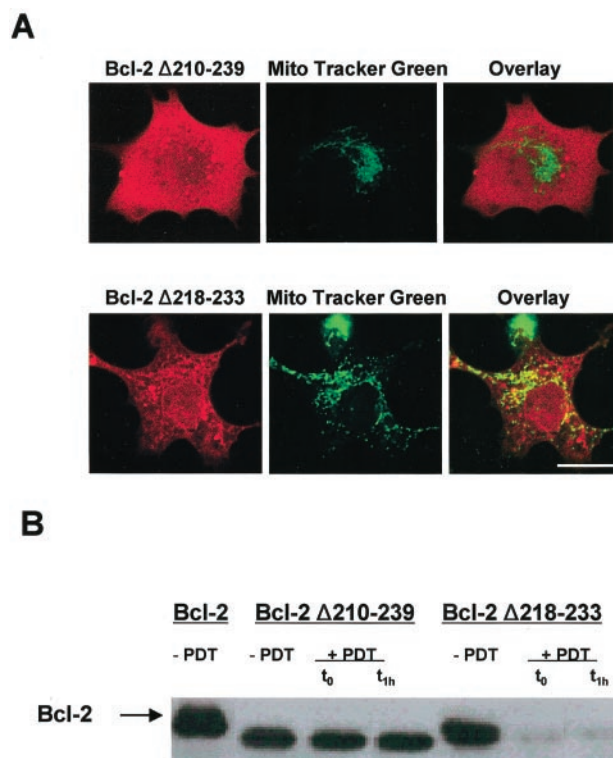
**Bcl-2 Mutants Missing Portions of the N-terminal Half of the Protein Respond to Pc 4-PDT Similarly to Wild-type Bcl-2**—We have reported that the antiapoptotic protein Bcl-2 disappears from Western blots immediately upon Pc 4-PDT (10). To elucidate the mechanism of the photodamage and reveal the target site, we constructed Bcl-2 mutants in which regions of the protein are deleted (Fig. 2, A and B). We used pcDNA4/HisMax plasmid, which encodes the Xpress<sup>TM</sup> epitope and a polyhistidine metal-binding tag at the N-terminal region of the multiple cloning site. We transiently transfected pcDNA4/HisMax-full-length human Bcl-2 (239 amino acids) and Bcl-2 mutants into DU-145 cells, which have very low endogenous levels of Bcl-2 (29). The subcellular localization of Bcl-2 in DU-145 cells was determined by immunocytochemical analysis with the anti-Xpress antibody and confocal microscopy. Bcl-2 co-localized with mitochondria and also localized to the nuclear envelope and other cellular organelles, as previously reported (17, 19, 28) (Fig. 3A). All of the mutants with deletions in the N-terminal half of Bcl-2 (mutants a, b, and c) localized in DU-145 cells similarly to wild-type Bcl-2. In control DU-145 cells transfected with empty vector, there was no detectable immunofluorescence signal above background (data not shown).

Twenty-four h after the transfection, we performed PDT. The dose of PDT in all experiments (200 nM Pc 4 plus 200 mJ/cm<sup>2</sup>) was demonstrated to produce  $92 \pm 2\%$  killing of untransfected DU-145 cells, as determined by clonogenic assay. The extent of photodamage was assessed by Western blot analysis. Similarly to the overexpressed wild-type Bcl-2, mutants with deletions in the N-terminal half of Bcl-2 (Bcl-2 ( $\Delta$ 33–54), Bcl-2 ( $\Delta$ 37–63) and Bcl-2 ( $\Delta$ 10–125)) were immediately photodamaged by Pc 4-PDT (Fig. 3B). One h after PDT, neither the wild-type Bcl-2 protein nor any of the mutant proteins had been restored to their previous levels (Fig. 3B). These results indicate that there is no essential target site of Pc 4-PDT in the N-terminal half of Bcl-2, and furthermore, Asp-34, a known caspase cleavage site (30–32), is not required for photodamage to Bcl-2 by Pc 4-PDT. Moreover, since essentially all of the Bcl-2 in the cell is damaged by the chosen dose of Pc 4-PDT, the results show that all of the sites of Bcl-2 localization are accessible for photodamage and that Pc 4 must reside within a few nm of Bcl-2 binding sites in the mitochondrial, ER, and nuclear membranes.



**FIG. 3. The subcellular localization and photosensitivity of mutant Bcl-2 having deletions within the N-terminal half of the protein.** DU-145 cells were transiently transfected with the pcDNA4/HisMax expression vector containing Bcl-2, Bcl-2 ( $\Delta 33-54$ ), Bcl-2 ( $\Delta 37-63$ ), or Bcl-2 ( $\Delta 10-125$ ). **A**, 24 h after transfection, subcellular localization of wild-type Bcl-2 and Bcl-2 mutant proteins was determined by imaging the fluorescence of Texas Red, and localization of mitochondria was determined by imaging the fluorescence of MitoTracker Green. Texas Red and MitoTracker Green images were overlaid to show the extent of co-localization in yellow. Scale bar, 5  $\mu\text{m}$ . **B**, other groups of cells were exposed to Pc 4-PDT (200 nM plus 200 mJ/cm<sup>2</sup> photoirradiation) 24 h after transfection. The Bcl-2 level was examined using a mouse monoclonal anti-Xpress tag antibody by Western blot analysis before (-PDT), immediately after ( $t_0$ ), and 1 h after ( $t_{1h}$ ) Pc 4-PDT. The control received Pc 4 but was not irradiated (-PDT).

*A Bcl-2 Mutant Missing the C-terminal 20 Amino Acids, Including the TM Domain, Was Not Destroyed by Pc 4-PDT, Whereas Retention of Part of the TM Domain Allows Photodamage to Occur*—A mutant (g) missing the C-terminal region, Bcl-2 ( $\Delta 210-239$ ), including the entire TM domain, was found throughout the cell in a diffuse nonmitochondrial pattern, as previously reported for a similar mutant (33) (Fig. 4A). Although some of the image of this mutant appeared punctate, there was no co-localization with MitoTracker Green. Mutant g displayed apparently complete resistance to photodamage by Pc 4-PDT, since there was no decrease in the amount of the protein either immediately after or 1 h after treatment of the

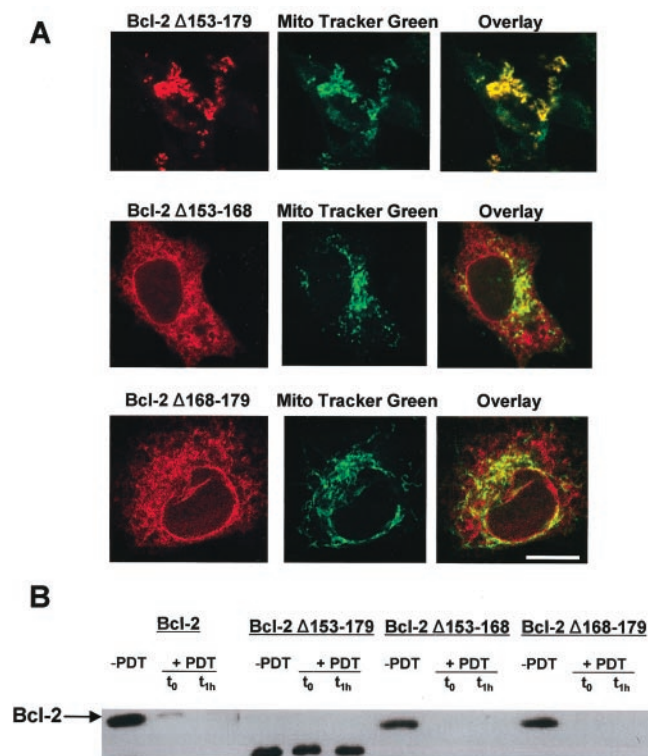


**FIG. 4. The subcellular localization and photosensitivity of mutant Bcl-2 missing portions of the C-terminal region of the protein.** DU-145 cells were transiently transfected with the pcDNA4/HisMax expression vector containing Bcl-2 ( $\Delta 210-239$ ) or Bcl-2 ( $\Delta 218-233$ ). **A**, 24 h after transfection, subcellular localization of the Bcl-2 mutant proteins was determined by imaging the fluorescence of Texas Red, and localization of mitochondria was determined by imaging the fluorescence of MitoTracker Green. Texas Red and MitoTracker Green images were overlaid to show the extent of co-localization in yellow. Scale bar, 5  $\mu\text{m}$ . **B**, other groups of cells were exposed to Pc 4-PDT (200 nM plus 200 mJ/cm<sup>2</sup> photoirradiation) 24 h after transfection. The Bcl-2 level was examined using a mouse monoclonal anti-Xpress tag antibody by Western blot analysis before (-PDT), immediately after ( $t_0$ ), and 1 h ( $t_{1h}$ ) after Pc 4-PDT. The control received Pc 4 but was not irradiated (-PDT).

cells with Pc 4-PDT (Fig. 4B). However, another C-terminal mutant (mutant h, Bcl-2 ( $\Delta 218-233$ )), was found in mitochondrial and nuclear membranes and was destroyed immediately after PDT (Fig. 4, A and B). Mutant h retains 4 amino acids (positions 234–237) within the TM domain, which is evidently sufficient for attachment to the membranes. These results indicate that for Pc 4-PDT to cause photodamage, membrane anchorage of the protein may be essential, which requires at least part of the C-terminal TM domain.

*Deletion of  $\alpha$ -Helices 5 and 6 in the Region between the BH1 and BH2 Domains Eliminates the Ability of Pc 4-PDT to Photodamage Bcl-2*—The region between the BH1 and BH2 domains, which contains two core hydrophobic  $\alpha$ -helices ( $\alpha_5$  and  $\alpha_6$ ), is required for membrane insertion and channel formation (17–19). Images of mutant d (Bcl-2 ( $\Delta 153-179$ )), which lacks both of the  $\alpha$ -helices, showed only punctate fluorescence in the cytoplasm that completely overlapped with the fluorescence of MitoTracker Green, indicating that Bcl-2 ( $\Delta 153-179$ ) localized to the mitochondrial membrane but not to the ER or nuclear envelope (Fig. 5A). Although Bcl-2 ( $\Delta 153-179$ ) retains the C-terminal TM domain and binds to mitochondria, it was not photodamaged by Pc 4-PDT (Fig. 5B). These results indicate that the two core hydrophobic regions between the BH1 and BH2 domains are required for Bcl-2 photodamage, and the C-terminal TM domain may not be a phototarget site. Interestingly, Bcl-2 proteins (mutants e and f) missing only one of the





**FIG. 5. The subcellular localization and photosensitivity of mutant Bcl-2 missing portions of the  $\alpha_5/\alpha_6$  region between the BH1 and BH2 domains.** DU-145 cells were transiently transfected with the pcDNA4/HisMax expression vector containing Bcl-2 ( $\Delta 153-179$ ), Bcl-2 ( $\Delta 153-168$ ), or Bcl-2 ( $\Delta 168-179$ ). A, 24 h after the transfection, subcellular localization of Bcl-2 mutant protein was determined by imaging the fluorescence of Texas Red, and localization of mitochondria was indicated by the fluorescence of MitoTracker Green. Texas Red and MitoTracker Green images were overlaid to show the extent of colocalization in yellow. Scale bar, 5  $\mu\text{m}$ . B, other groups of cells were exposed to PDT (200 nM Pc 4 plus 200  $\text{mJ}/\text{cm}^2$  photoirradiation) 24 h after the transfection. The Bcl-2 level was examined using a mouse monoclonal anti-Xpress tag antibody by Western blot analysis before (-PDT), immediately after ( $t_0$ ), and 1 h after ( $t_{1h}$ ) Pc 4-PDT. The control received Pc 4 but was not irradiated (-PDT).

$\alpha$ -helices (Bcl-2 ( $\Delta 153-168$ ) and Bcl-2 ( $\Delta 168-179$ )) behaved like wild-type Bcl-2 (Fig. 5A). Figs. 2 and 7 show that the 16 amino acids (positions 153–168) deleted in mutant e correspond to part of the  $\alpha_5$  region, and the 12 amino acids (positions 168–179) deleted in mutant f correspond to part of the  $\alpha_6$  region. Both of these proteins localized similarly to wild-type Bcl-2 and could be destroyed by Pc 4-PDT (Fig. 5, A and B). These results indicate that both  $\alpha_5$ - and  $\alpha_6$ -helices may be required for attachment of the protein to the ER and nuclear membrane but that either the  $\alpha_5$  or the  $\alpha_6$  domain is sufficient for photodamage through attachment to or insertion into the mitochondrial membrane.

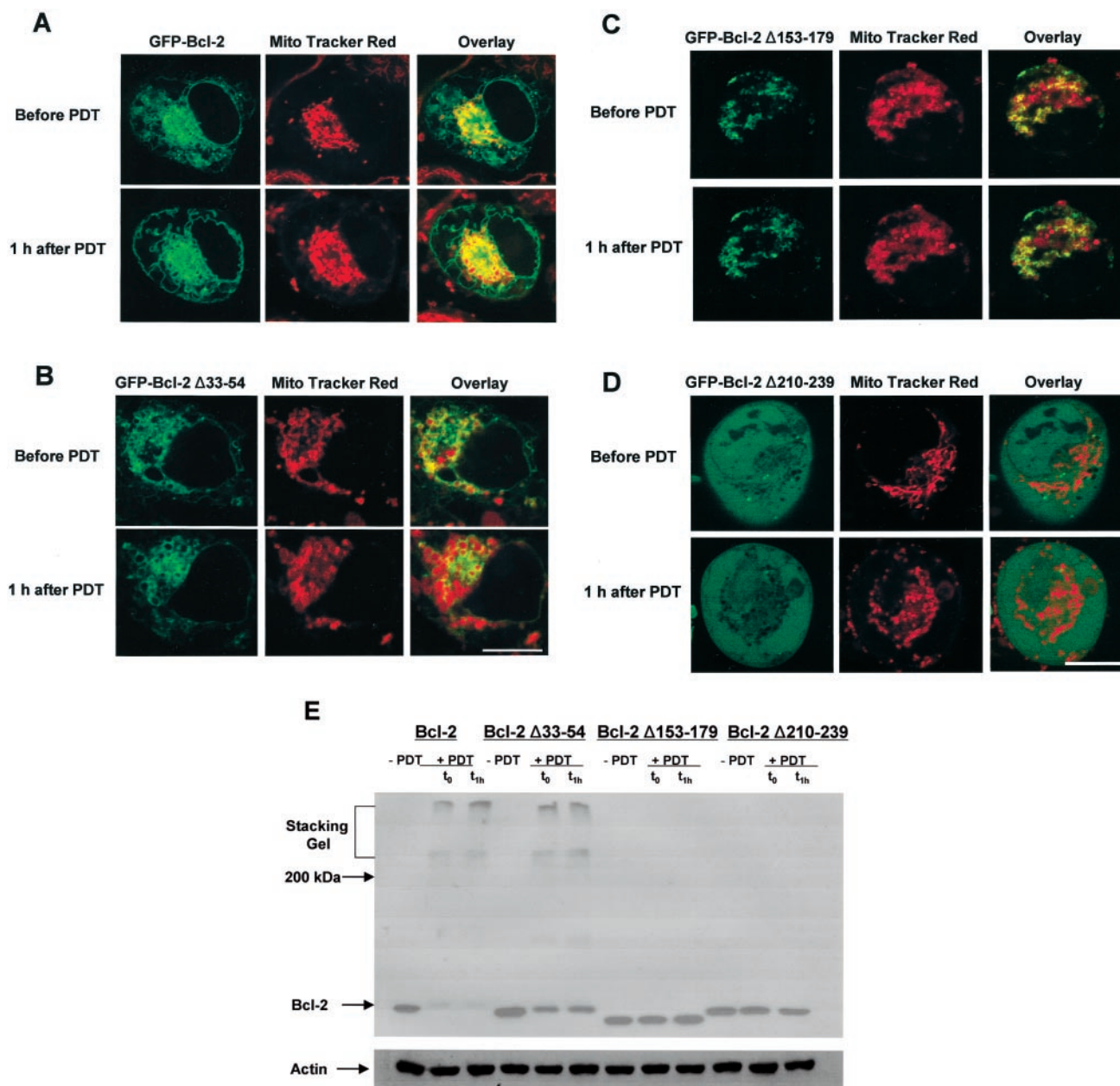
**Observation of GFP-Bcl-2 Fluorescence in Live Cells Revealed That Photodamaged Bcl-2 Remains at Its Original Sites in the Nuclear Envelope, ER, and Mitochondrial Membrane after Pc 4-PDT**—Until this point, photodamage has been monitored by Western blot analysis, which reveals that immediately after Pc 4-PDT, there is a nearly complete loss of the 26-kDa protein. Since it is unlikely that this response reflects the immediate disappearance of the protein from the cells, we sought further clarification of the photodamage response by monitoring the fate of GFP-tagged Bcl-2 in live cells following Pc 4-PDT. Thus, we constructed GFP-Bcl-2 and GFP-Bcl-2 mutants and examined the localization of the GFP fusion proteins by confocal microscopy before and after PDT. GFP-Bcl-2 localized to the nuclear envelope, ER, and mitochondria (Fig. 6A), as previ-

ously observed by immunohistochemical analysis of Xpress-tagged Bcl-2 in Fig. 3A. One h after Pc 4-PDT, GFP-Bcl-2 remained in the same locations with approximately the same fluorescence intensity. It is also apparent that the mitochondria had swollen by 1 h after Pc 4-PDT, as we had previously found in A431 cells (7), and some of the GFP-Bcl-2 clearly outlines the swollen mitochondria (Fig. 6A). GFP-Bcl-2 ( $\Delta 33-54$ ), which was photodamaged by Pc 4-PDT (Fig. 3B), also did not disappear from its normal binding sites after PDT (Fig. 6B); this mutant revealed the same localization pattern as wild-type GFP-Bcl-2. We observed neither aggregation of organelles nor large complexes of GFP-Bcl-2, despite greater irregularity in the shape of the nuclear membrane and swelling of the mitochondria after PDT (Fig. 6, A and B).

The two mutants that did not suffer from photodamage, Bcl-2 ( $\Delta 153-179$ ) and Bcl-2 ( $\Delta 210-239$ ) (Figs. 4B and 5B), were also examined as GFP-tagged proteins by confocal microscopy in live cells (Fig. 6, C and D). Similar to the photodamageable Bcl-2 species (Fig. 6, A and B), the localization and intensity of the nonphotodamageable mutants were identical before and after PDT (Fig. 6, C and D), and the localizations were similar to those observed in Figs. 4A and 5A. For the four GFP-Bcl-2 species examined in Fig. 6, images of GFP intensity and localization obtained immediately after Pc 4-PDT were not different from those at 1 h after PDT, except for less mitochondrial swelling at the early time (data not shown). These results indicate that Pc 4-PDT-damaged Bcl-2 remains at its previous sites in the cell.

**Photodamage to Bcl-2 Is Observed on Western Blots as the Disappearance of the Control Band and the Appearance of High Molecular Weight Bands**—The above data demonstrate that photodamaged Bcl-2 remains at its original sites in cells but is not found at its normal position on Western blots. These observations suggest that there has been a photochemical change in the protein that alters its electrophoretic mobility. Previously, we found evidence for high molecular weight complexes containing Bcl-2 in Western blots of Pc 4-PDT-treated Chinese hamster ovary cells (10). In order to further characterize the photodamage, some of the previously analyzed cell lysates (Figs. 3B, 4B, and 5B) were reanalyzed; however, for these experiments, proteins in both the running and stacking gels were transferred to polyvinylidene difluoride membranes, and the entire blot was evaluated using the mouse monoclonal anti-Xpress antibody (Fig. 6E). Essentially the same results were obtained for cells overexpressing GFP-wild-type Bcl-2 or GFP-Bcl-2 mutant, when the entire gel was transferred and evaluated with the anti-GFP antibody (data not shown).

As observed previously (Figs. 3B, 4B, and 5B), photodamage to wild-type Bcl-2 and to Bcl-2 ( $\Delta 33-54$ ) was revealed as a reduction in intensity of the control band, whereas there was no PDT-induced change in intensity for the nonphotodamageable species, Bcl-2 ( $\Delta 153-179$ ) and Bcl-2 ( $\Delta 210-239$ ). In addition, it is apparent that photodamage was accompanied by the appearance of high molecular mass (>250-kDa) bands in the regions corresponding to the top of the running gel and the stacking gel, and these were observed in samples taken both immediately after and 1 h after PDT (Fig. 6E). These results indicate that Pc 4-PDT forms large cross-linked complexes of Bcl-2 or Bcl-2 ( $\Delta 33-54$ ), but not of Bcl-2 ( $\Delta 153-179$ ) or Bcl-2 ( $\Delta 210-239$ ). The large complexes include Bcl-2 and possibly neighboring proteins and are sufficiently large that in many cases, they are unable to enter the gel. Therefore, photodamaged Bcl-2 seems to have disappeared only insofar as it has been converted from its native size into large complexes, but it does not disappear from the cells. The absence of cross-link formation with Bcl-2 ( $\Delta 153-179$ ) and Bcl-2 ( $\Delta 210-239$ ) (Fig.



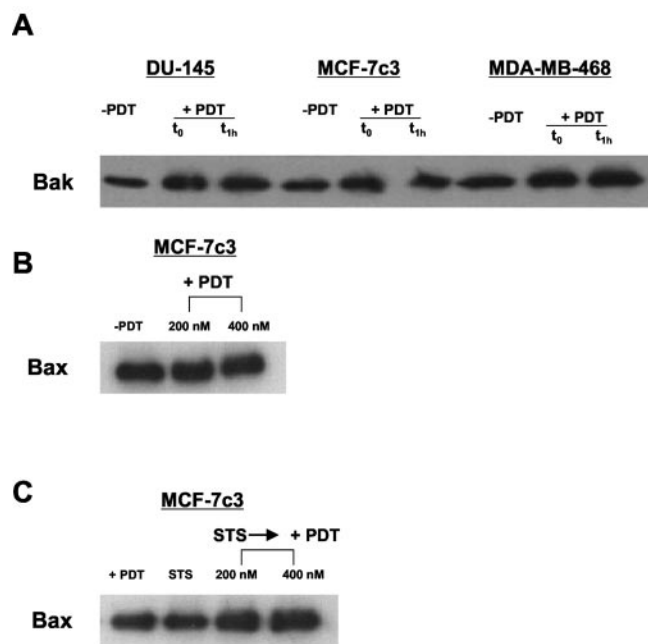
**FIG. 6. The subcellular localization and photosensitivity of GFP-Bcl-2 and GFP-Bcl-2 mutants before and 1 h after PDT.** DU-145 cells were transiently transfected with an expression vector encoding Bcl-2, Bcl-2 ( $\Delta 33-54$ ), Bcl-2 ( $\Delta 153-179$ ), or Bcl-2 ( $\Delta 210-239$ ) as a fusion protein with GFP at the N terminus. *A-D*, 8 h after the transfection, 200 nM Pc 4 was added to the cultures, which were further incubated for 16 h and incubated with 100 nM MitoTracker Red for 45 min. Then cultures were irradiated with 200 mJ/cm<sup>2</sup> red light. In living DU-145 cells, localizations of Bcl-2 and Bcl-2 mutants were determined by observing GFP fluorescence, and localizations of mitochondria were determined by observing fluorescence of MitoTracker Red. GFP and MitoTracker Red images were overlaid to show colocalization in yellow. In cells transfected with GFP-Bcl-2 (*A*), GFP-Bcl-2 ( $\Delta 33-54$ ) (*B*), GFP-Bcl-2 ( $\Delta 153-179$ ) (*C*), or GFP-Bcl-2 ( $\Delta 210-239$ ) (*D*), images were collected before and 1 h after PDT. Scale bar, 5  $\mu$ m. *E*, whole cell lysates used in previous experiments (Figs. 3*B*, 4*B*, and 5*B*) were loaded to 10% SDS-PAGE gels. Proteins in both the running and stacking gels were transferred to polyvinylidene difluoride membranes. The Bcl-2 level was examined using a mouse monoclonal anti-Xpress antibody by Western blot analysis before ( $-PDT$ ), immediately after ( $t_0$ ), and 1 h after ( $t_{1h}$ ) PDT. The membrane was reprobed with anti-actin to control for loading. Control cells received Pc 4 but were not irradiated ( $-PDT$ ).

6*E*) suggests that the membrane anchorage domains are required for formation of the membrane-associated protein complexes that are susceptible to photocross-linking. In the case of Bcl-2 ( $\Delta 210-239$ ), the absence of membrane anchorage (Fig. 6*D*) prevents photodamage via photoirradiated membrane-localized Pc 4. However, in the case of Bcl-2 ( $\Delta 153-179$ ), which binds to the mitochondrial membrane (Fig. 6*C*), the absence of photodamage reveals an important role for the  $\alpha_5/\alpha_6$  domain. Either this domain is the direct target of photodynamic damage, or it is needed to form the photosensitive structure.

*Pc 4-PDT Does Not Directly Damage the Singlet Oxygen-sensitive Amino Acids within the  $\alpha_5$  and  $\alpha_6$   $\alpha$ -Helices*—PDT generates singlet oxygen and other reactive oxygen species at photosensitizer binding sites (34, 35). In order to determine whether or not the  $\alpha_5$  and  $\alpha_6$   $\alpha$ -helices are damaged directly by Pc 4-PDT-produced singlet oxygen, we modified Bcl-2 mutants e and f to construct mutants i and j by site-directed mutagenesis. Fig. 7 shows the amino acid sequence of the relevant parts of the  $\alpha_5/\alpha_6$  region of Bcl-2, in which the hydrophobic amino acids are coded red, and the <sup>1</sup>O<sub>2</sub>-reactive amino acids (Cys, Phe,







**FIG. 9. Resistance of Bak and Bax to photodamage by Pc 4-PDT.** *A*, DU-145 cells, MCF-7c3 cells, and MBA-MB-468 cells were exposed to 200 nM Pc 4. After 16 h, the cells were irradiated with red light (200 mJ/cm<sup>2</sup>). Immediately after or 1 h after PDT, cells were collected, and Bak levels were examined by Western blot analysis. *B*, MCF-7c3 cells were treated with either 200 or 400 nM Pc 4. After 16 h, the cells were irradiated with red light (200 mJ/cm<sup>2</sup>). Immediately after PDT, cells were harvested, and Bax levels were analyzed by Western blot. *C*, MCF-7c3 cells were exposed to 200 or 400 nM Pc 4. After 12 h, 100 nM staurosporine was added to the cultures, and 4 h later, the cells were irradiated with red light (200 mJ/cm<sup>2</sup>). Immediately after PDT, the cells were collected, and Bax levels were analyzed by Western blot. Controls included cells treated only with PDT or only with staurosporine (STS).

photodynamic action and does not require protease or other enzymatic activity.

In the present study, we found that overexpressed tagged wild-type Bcl-2 protein and overexpressed GFP-Bcl-2 protein, which localized to the mitochondria, ER, and nuclear envelope (Figs. 3A and 6A), were photodamaged immediately and did not recover by 1 h after PDT. Since essentially all of the Bcl-2 was photodamaged, our data indicate that Pc 4-PDT damages not only mitochondrial membranes but also the ER and nuclear envelope. Kvam *et al.* (40) have reported that PDT using mesotetra-(3-sulfonatophenyl)porphyrin damaged the nuclear membrane and induced DNA repair, although much subsequent work has indicated that DNA is not an important target for cell killing by PDT (41, 42). Pc 4 binds to the same membrane systems that house Bcl-2 and therefore may be especially well positioned to damage all cellular Bcl-2, not just the mitochondrially bound protein.

The subcellular localization of Pc 4 is similar in DU-145 cells as in other cell types (6, 7) (*i.e.* cytoplasmic membranes, including mitochondria, ER, and nuclear envelope) (Fig. 1). The reactive oxygen species produced by PDT, especially singlet oxygen, are highly reactive and therefore cause photooxidative damage to proteins and lipids that reside within a few nm of photosensitizer binding sites (34, 35). A major conclusion of the present study is that the photodamage and cross-linking of Bcl-2 are absolutely dependent upon anchorage of the protein in membranes. We found that two membrane anchorage domains of Bcl-2 were required for efficient photodamage. One is the region between the BH1 and BH2 domains, corresponding to the  $\alpha_5$ - and  $\alpha_6$ -helices, and the other is the C-terminal TM domain, especially four amino acids (positions 234–237) within

the TM domain. The C-terminal TM domain is required for Bcl-2 to be photodamaged and to become cross-linked. However, mutant h, which is missing 15 amino acids from the TM domain, was photodamaged (Fig. 4B). These results suggest that the missing 15 amino acids (positions 219–233) are not the target sites of Pc 4-PDT, and the four retained amino acids (positions 234–237) are sufficient for Bcl-2 photodamage. If those four amino acids were the binding sites of Pc 4 and the targeted region for Pc 4-PDT, mutant d, Bcl-2 ( $\Delta 153$ –179), which has the full C-terminal TM domain and localizes to mitochondria, would have been photodamageable and capable of forming cross-links upon PDT. Moreover, other Bcl-2 family members that have a similar C-terminal TM domain to that of Bcl-2 should also have been photodamaged. Therefore, we conclude that the four amino acids within the C-terminal TM are sufficient to attach Bcl-2 to the membrane in a photodamageable form, but that region is not the target site of Pc 4-PDT.

We hypothesize that the region between the BH1 and BH2 domains, corresponding to the  $\alpha_5$ - and  $\alpha_6$ -helices, is the more important region for forming the photodamageable structure. Furthermore, Pc 4 may bind to the membrane close to the region where Bcl-2 is located. Muchmore *et al.* (16) have reported that Bcl-2 forms pores in the cytoplasmic membranes where it localizes, and the region between the BH1 and BH2 domains of Bcl-2 is inserted into the membranes and regulates cell death. Mutations within that region, which are similar to Bcl-2 ( $\Delta 153$ –179), abrogate the antiapoptotic activity (18–20). Some reports have shown that mutants missing the C-terminal TM domain but retaining the region of the  $\alpha_5$ - and  $\alpha_6$ -helices have antiapoptotic activity (14, 15). We suggest that the  $\alpha_5/\alpha_6$  region of Bcl-2 is very important to its antiapoptotic activity, but its role in determining the subcellular localization of Bcl-2 is still not well defined. Conus *et al.* (33) reported that Bcl-2 missing the region between the BH1 and BH2 domains (*i.e.* similar to Bcl-2 ( $\Delta 153$ –179)) localized to mitochondria. They suggested that the mutant Bcl-2 perhaps had a higher affinity for mitochondria than for other membranes, like ER and nuclear envelope. Wang *et al.* (26) showed that ER-targeted Bcl-2 prevented cell death induced by Bax overexpression, whereas mitochondrion-targeted Bcl-2 was necessary for Bax-induced cell death. In our study, Bcl-2 missing the  $\alpha_5/\alpha_6$  region attached to the mitochondria but not to the ER or the nuclear membrane (Fig. 5B).

An important question is whether the photodamaged Bcl-2 retains its antiapoptotic function. We were not able to observe morphologically typical apoptosis (*i.e.* chromatin condensation and fragmentation) in DU-145 cells after PDT because of the absence of Bax from these cells. However, the cells die after PDT without undergoing typical apoptosis, as determined by clonogenic assay.<sup>2</sup> Moreover, DU-145 cells that stably overexpress Bcl-2 have a similar sensitivity to Pc 4-PDT as those expressing only endogenous Bcl-2. Although the absence of a differential photosensitivity might be attributed to the inability of DU-145 cells to up-regulate Bax (11), the same observation has been made in MCF-7 cells that express adequate levels of Bax. We suggest that Pc 4-PDT-induced photodamage eliminates the native Bcl-2 structure and thereby its antiapoptotic activity. Since all Bcl-2 is photodamaged, the pre-PDT level of Bcl-2 may not be relevant to determining the cell's sensitivity to Pc 4-PDT. Support for this idea derives from observations (43, 44) that overexpressed Bcl-2 does not protect cells against PDT-induced release of cytochrome *c*. Further work will be required to assess the function of photodamaged Bcl-2. We

<sup>2</sup> L. Y. Xue, J. Usuda, S. Chiu, and N. L. Oleinick, manuscript in preparation.



have begun experiments to evaluate by flow cytometry the ability of transiently expressed Bcl-2 and mutants to modify Pc 4-PDT-induced apoptosis.

The region between the BH1 and BH2 domains of Bcl-2 is very similar to those of Bak and Bax (16). The proapoptotic protein Bak localizes to the mitochondrial membrane but is not destroyed by Pc 4-PDT (Fig. 9A). One possible reason for the resistance of Bak to photodamage is that it may reside in the membrane at a site far from the sites of singlet oxygen generation. Another possibility is suggested by the observations of Griffiths *et al.* (38) that the Bak N-terminal domain regulates the conformation of the BH1 domain, and apoptotic signals promote sequential changes in the N-terminal and BH1 domains. In that case, conformational regulation of the  $\alpha_5/\alpha_6$  region of Bak by the N-terminal domain may prevent insertion into the membrane.

Based on analysis of the three-dimensional structure of Bax, its normal conformation is reported to involve an interaction between the BH3 domain and the N terminus; in response to an apoptotic signal, Bax undergoes a conformational change, which results in rotation of the BH3 domain away from the N terminus (45–48). Eskes *et al.* (49) and Antonsson *et al.* (50) demonstrated that in apoptotic cells, Bax was not only attached to the mitochondrial membrane but also inserted into it. However, despite having an  $\alpha_5/\alpha_6$  region similar to that of Bcl-2 and apoptosis-induced translocation to the mitochondria in response to staurosporine, Bax was not photodamaged by Pc 4-PDT (Fig. 7C) (10) or by PDT with AlPc (11).

If Pc 4 were homogeneously distributed in the membranes, any membrane-inserted protein would be susceptible to photodamage. However, other mitochondrial membrane proteins, including the voltage-dependent anion channel and the adenine nucleotide translocator, were not photodamaged by Pc 4-PDT (10). Thus, the cross-linking is a relatively specific response of Bcl-2 to Pc 4-PDT, which may result from a greater photosensitivity of the Bcl-2 complexes or greater exposure of those complexes to PDT-generated singlet oxygen.

We conclude that the  $\alpha_5/\alpha_6$  region of Bcl-2 is not the direct target of Pc 4-PDT but contributes to photodamage by forming essential interactions with other domains of Bcl-2. From our photobiological analysis of Bcl-2 family members, it appears that cross-link formation of Bcl-2 might play an important role in the induction of apoptosis by PDT, either by blocking its antiapoptotic function or by forming a proapoptotic form of Bcl-2.

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