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Characterization of the signal that directs Bcl-x_L, but not Bcl-2, to the mitochondrial outer membrane

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It is assumed that the survival factors Bcl-2 and Bcl-x_L are mainly functional on mitochondria and therefore must contain mitochondrial targeting sequences. Here we show, however, that only Bcl-x_L is specifically targeted to the mitochondrial outer membrane (MOM) whereas Bcl-2 distributes on several intracellular membranes. Mitochondrial targeting of Bcl-x_L requires the COOH-terminal transmembrane (TM) domain flanked at both ends by at least two basic amino acids. This sequence is a bona fide targeting signal for the MOM as it confers specific mitochondrial localization to soluble EGFP. The signal is present in numerous proteins known to be directed to the MOM. Bcl-2 lacks the signal and therefore localizes to several intracellular membranes. The COOH-terminal region of Bcl-2 can be converted into a targeting signal for the MOM by increasing the basicity surrounding its TM. These data define a new targeting sequence for the MOM and propose that Bcl-2 acts on several intracellular membranes whereas Bcl-x_L specifically functions on the MOM.

Introduction

The Bcl-2 family of proteins are central regulators of apoptosis because they integrate diverse survival and death signals that are generated outside and inside the cell (Cory and Adams, 2002). The family is subdivided into two classes, anti-apoptotic members such as Bcl-2 and Bcl-x_L (Bcl-2-like survival factors), which protect cells from apoptosis, and pro-apoptotic members such as Bax and Bak (Bax-like death factors) and the BH3-only death proteins, which trigger or sensitize cells for apoptosis (Antonsson, 2001; Puthalakath and Strasser, 2002). An important, although not exclusive, action of Bcl-2 family members is to control mitochondrial membrane permeability. In response to various apoptotic stimuli, the mitochondrial outer membrane (MOM)* is perforated without disrupting the general integrity of the organelle (von Ahsen et al., 2000). As a consequence, proteins that are safely sequestered in the mitochondrial intermembrane space in healthy cells, leak into the cytoplasm where they participate in caspase-dependent and -independent apoptosis signaling (Desagher and Martinou, 2000).

Whereas Bax-like factors increase mitochondrial permeability, Bcl-2–like factors block this process (Antonsson, 2001; Cory and Adams, 2002). The two protein classes can neutralize each other by heterodimerization but this does not entirely explain their function (Knudson and Korsmeyer, 1997). Recent studies showed that the BH3-only proteins are required for the apoptotic regulation of Bcl-2– and Bax-like factors (Puthalakath and Strasser, 2002). These proteins act as "sensors" for apoptotic stimuli at different locations within the cells. In response to a defined stimulus, a particular BH3-only protein is modified by proteolysis or posttranslational modification, leaves its intracellular site, and travels to the mitochondrial membrane where it can choose two fates. Either it interacts, via its BH3 domain, with Bcl-2–like survival factors to release a pro-apoptotic protein, or it interacts with Bax-like factors to stimulate their oligomerization and/or membrane insertion. In both cases, Bax and Bak are activated to trigger MOM perforation by a mechanism that has yet to be identified. On the other hand, Bcl-2 and Bcl-x_L inhibit cell death when sufficiently expressed to sequester BH3-only proteins, Bax-like factors, and other pro-apoptotic molecules (Cory and Adams, 2002).

It is thought that Bcl-2 and Bcl-x_L are redundant in their capacity to protect cells from apoptosis (Chao et al., 1995). Both proteins block BH3-only and Bax/Bak-mediated MOM perforation and may therefore primarily function on mitochondria. However, Bcl-2 also localizes to the nuclear

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*Abbreviations used in this paper: IVTT, in vitro transcription–translation; MOM, mitochondrial outer membrane; TM, transmembrane.

Key words: apoptosis; Bcl-2; Bcl-x_L; mitochondria; ER

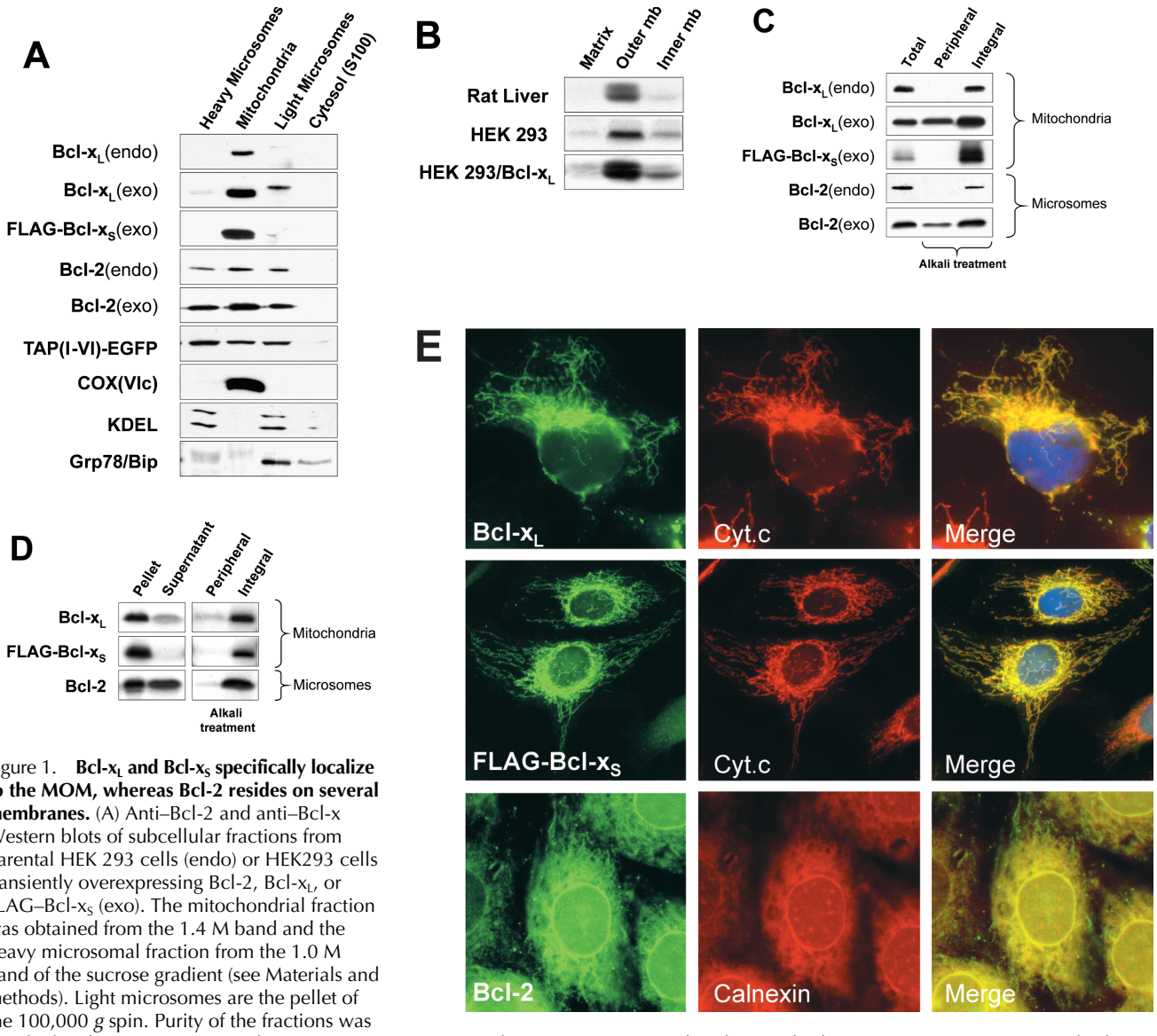

Figure 1. **Bcl-x_L and Bcl-x_S specifically localize to the MOM, whereas Bcl-2 resides on several membranes.** (A) Anti–Bcl-2 and anti–Bcl-x Western blots of subcellular fractions from parental HEK 293 cells (endo) or HEK293 cells transiently overexpressing Bcl-2, Bcl-x_L, or FLAG–Bcl-x_S (exo). The mitochondrial fraction was obtained from the 1.4 M band and the heavy microsomal fraction from the 1.0 M band of the sucrose gradient (see Materials and methods). Light microsomes are the pellet of the 100,000 *g* spin. Purity of the fractions was checked with anti-grp78/Bip and anti-KDEL (microsomes) and anti–COX-VIc (mitochondria) antibodies. TAP(I-VI)–EGFP contains the first six membrane-spanning regions of the antigen peptide transporter I (TAP I) fused to EGFP. This protein specifically spans the ER membrane and is detected by anti-GFP Western blotting after transient transfection. (B) Anti–Bcl-x Western blots of mitochondrial matrix, inner membrane (mb), and outer membrane fractions of rat liver, HEK293 cells, and HEK293 cells transiently overexpressing Bcl-x_L (HEK293/Bcl-x_L). (C) Anti–Bcl-2 and anti–Bcl-x Western blots of mitochondria or microsomes from parental HEK293 cells or HEK293 cells overexpressing Bcl-2 or Bcl-x_L or FLAG–Bcl-x_S, extracted directly with detergent (total) or first treated with sodium carbonate (pH 12, peripheral) and then extracted with detergent (integral). (D) Autoradiography of [^{35}S]methionine-labeled, in vitro–transcribed/translated (IVTT) Bcl-2, Bcl-x_L, or FLAG–Bcl-x_S inserted (alkali resistant, integral) or loosely attached (alkali extractable, peripheral) to mitochondria or microsomes (pellet), or remaining in the supernatant after spinning off the organelles. (E) Anti–Bcl-2 and anti–Bcl-x immunofluorescence analysis of R6 cells transiently overexpressing Bcl-x_L, FLAG-Bcl-x_S, or Bcl-2 (green). Whereas both Bcl-x_L and FLAG–Bcl-x_S colocalize with the mitochondrial marker cytochrome c (Cyt.c, red), Bcl-2 colocalizes with the ER marker calnexin (red). Nuclei were stained with Hoechst 33342 (blue in the merge).

envelope and the membrane of the ER (Krajewski et al., 1993; Givol et al., 1994; Janiak et al., 1994; Lithgow et al., 1994; Conus et al., 2000b), where it can theoretically sequester pro-apoptotic molecules in a similar way as on mitochondria. Indeed, if Bcl-2 is artificially targeted to the ER, it still protects cells from apoptosis (Zhu et al., 1996; Hacki et al., 2000). A recent study revealed that specific targeting of Bcl-2 to mitochondria converts it into a pro-apoptotic molecule (Wang et al., 2001), suggesting that mitochondria may not be the preferred site for the survival action of Bcl-2.

Both Bcl-2 and Bcl-x_L are tail-anchored proteins, i.e., they contain a COOH-terminal hydrophobic helix that functions as a membrane insertion device (transmembrane [TM] domain) (Chen-Levy and Cleary, 1990; Nguyen et al., 1993; Janiak et al., 1994; Kim et al., 1997). Proteins that are tail anchored to the MOM or the ER, such as the MOM translocases TOM22 (Egan et al., 1999) and TOM5 (Horie et al., 2002) or the microsomal form of cytochrome b5 (De Silvestris et al., 1995), use their TM anchor also as a targeting signal. Although targeting signals for the translocation of proteins across the ER membrane or the inner mitochon-

drial membrane are well known (Neupert, 1997), those that target proteins to the MOM have only recently begun to be unveiled. A putative targeting signal for the MOM may be a hydrophobic TM region with a particular length and hydrophobicity followed by one to two basic amino acids (TMB) (Mihara, 2000; Wattenberg and Lithgow, 2001). As both Bcl-2 and Bcl-x_L contain one to two basic amino acids after their TM region, it was proposed that they are both targeted to the MOM (Kuroda et al., 1998; Everett et al., 2000). However, localization studies have been mostly performed under nonphysiological conditions where Bcl-2 and Bcl-x_L were overexpressed. It has therefore remained elusive whether endogenous Bcl-2 and Bcl-x_L distribute evenly or are specifically targeted to the MOM. Moreover, there has not been a systematic analysis on the signal sequence that directs these proteins to organelles.

Here we perform an extensive mutagenesis analysis in the COOH terminus of Bcl-2 and Bcl-x_L. We show that Bcl-x_L contains a particular mitochondrial signal sequence in this region whereas Bcl-2 does not. This signal requires two basic amino acids at both ends of the TM domain. Importantly, we show that not only overexpressed but also endogenous Bcl-x_L and Bcl-2 localize to different subcellular compartments. It is proposed that Bcl-x_L specifically acts on mitochondria whereas Bcl-2 can also control ER events associated with apoptosis.

Results

Bcl-x_L/x_S are integral proteins of the MOM whereas Bcl-2 inserts into several membranes

Endogenous and overexpressed Bcl-x_L were enriched in mitochondria when purified subcellular fractions of HEK293 cells were analyzed on anti–Bcl-x immunoblots (Fig. 1 A). A protein band with a slightly higher molecular mass was detected in the light microsomal fraction of cells overexpressing Bcl-x_L (Fig. 1 A). In certain cell types, Bcl-x_L also appeared in the cytosol, especially when the protein was overexpressed (unpublished data). Immunoblots with organelle-specific marker antibodies revealed that the subcellular fractions were pure (Fig. 1 A). Nuclear fractions are not shown because they could not be reproducibly deprived of cosedimenting or aggregated mitochondria and unlysed cells. A further subfractionation of HEK293 and rat liver mitochondria showed that the major proportion of Bcl-x_L resided in the MOM (Fig. 1 B). Endogenous Bcl-x_L was stably integrated into the mitochondrial membrane because it could only be extracted with detergent (integral) (Fig. 1 C). By contrast, some of the overexpressed Bcl-x_L was loosely (peripherally) attached to this membrane. When Bcl-x_L was in vitro transcribed/translated and added to purified mitochondria, most of the protein was recovered in the mitochondrial pellet in a stably inserted form (Fig. 1 D). The pro-apoptotic splice variant Bcl-x_S, which is identical to Bcl-x_L but lacks the BH1/BH2 regions (Boise et al., 1993), also accumulated in mitochondria after ectopic expression in HEK293 cells (Fig. 1 A) and in vitro translation (Fig. 1 D) and inserted even better into the mitochondrial membrane than Bcl-x_L (Fig. 1 C). The endogenous Bcl-x_S protein could not be studied because it was not expressed at detectable levels in 10 different cell lines (unpublished data). Strikingly, in contrast to Bcl-x_L and Bcl-x_S, endogenous and overexpressed Bcl-2 were found in all intracellular membrane fractions (Fig. 1 A). The mitochondrial portion of Bcl-2 may be even overestimated, as some heavy microsomal membranes could not be entirely separated from mitochondria, as evidenced by the ER membrane–specific marker TAP(I-VI)–EGFP (Vos et al., 2000) (Fig. 1 A). This fraction did not contain the luminal grp78/Bip and KDEL proteins and may consist of microsomal membranes that are fused to mitochondria as recently proposed (Landolfi et al., 1998; Prinz et al., 2000). Bcl-2 integrated into the microsomal membrane in a similar way as Bcl-x_L integrated into mitochondria. Although endogenous Bcl-2 was never cytosolic and stably inserted into membranes (Fig. 1, A and C), some of the overexpressed form loosely attached to microsomes (Fig. 1 C) and remained in the supernatant after in vitro transcription/translation (Fig. 1 D). Immunofluorescence analysis of R6 (Fig. 1 E) or HeLa cells (unpublished data) confirmed our in vitro data. Whereas Bcl-2 colocalized with the ER protein calnexin on nuclear/ER membrane structures, Bcl-x_L and Bcl-x_S were consistently immunodetected in filamentous structures that colocalized with the mitochondrial markers TOM20, COX, or cytochrome c (Fig. 1 E). Our data show that Bcl-x_L/x_S and Bcl-2 are primarily membrane-inserted proteins, but with different targeting specificities.

The TMB region is important for the membrane targeting of Bcl-x_L/x_S and Bcl-2

What determines the specific targeting of Bcl-x_L/x_S to the MOM and why is Bcl-2 incapable of doing so? Targeting sequences for the MOM have recently been identified in proteins of the TOM complex, monoamine oxidase A/B, VAMP-1B, and members of the Bcl-2 family (for reviews see Mihara, 2000; Wattenberg and Lithgow, 2001). These sequences are present at either the NH_2 or COOH termini of the proteins and consist of a hydrophobic, α-helical TM region followed by one or two basic amino acids (TMB). Both Bcl-x_L/x_S and Bcl-2 contain a typical TMB domain at their COOH terminus (Fig. 2 A). To investigate the role of the TMB of Bcl-x_L/x_S and Bcl-2 in (mitochondrial) membrane targeting, this region was deleted (Fig. 2 B) and the tailless proteins transiently expressed in R6, HeLa, and HEK293 cells. Bcl-2ΔTMB, Bcl-x_LΔTMB, and FLAG-tagged Bcl-x_S-ΔTMB proteins were all immunodetected in cytosolic fractions and exhibited a diffuse cellular staining (Fig. 3, A and C). For Bcl-2ΔTMB, we also noticed a staining of the nuclear envelope (Fig. 3 C), and part of the protein copurified with a light microsomal fraction (Fig. 3 A). In addition, a portion of in vitro–translated Bcl-2ΔTMB was recovered in the pellet after incubation with microsomes (Fig. 3 B). However, the protein was only peripherally attached to membranes (Fig. 3 A), indicating that its membrane association was possibly a side effect of overexpression. Bcl-x_LΔTMB and FLAG–Bcl-x_S-ΔTMB were even less detected in membrane fractions than Bcl-2ΔTMB (Fig. 3, A and B), and the type of membrane was microsomal rather than mitochondrial, indicating that the tailless proteins lacked specific MOM targeting (Fig. 3 A).

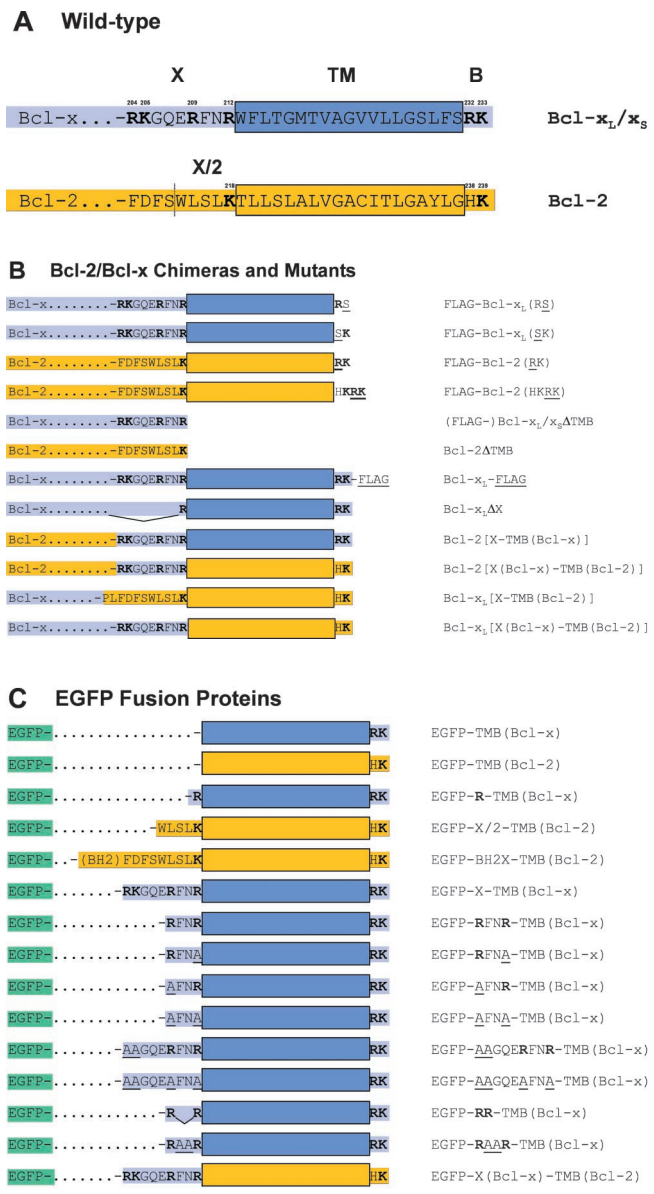

Figure 2. **Schematic representation of Bcl-2, Bcl-x$_L$, Bcl-x$_S$, their mutants, and the EGFP fusion constructs.** Schematic structure and amino acid sequences of (A) the COOH-terminal parts of wild-type Bcl-2 (yellow) and Bcl-x$_L$/x$_S$ (blue), including the 19–amino acid-long TM domain, flanked by one to two basic amino acids at one end (B) and the X or X/2 domain (half of the X domain) at the other end (basic amino acids are numbered and indicated in bold); (B) the COOH-terminal parts of Bcl-2 and Bcl-x$_L$/x$_S$ mutants (point mutations and insertions are underlined); (C) the COOH-terminal mutants of Bcl-2 and Bcl-x fused to the COOH terminus of EGFP.

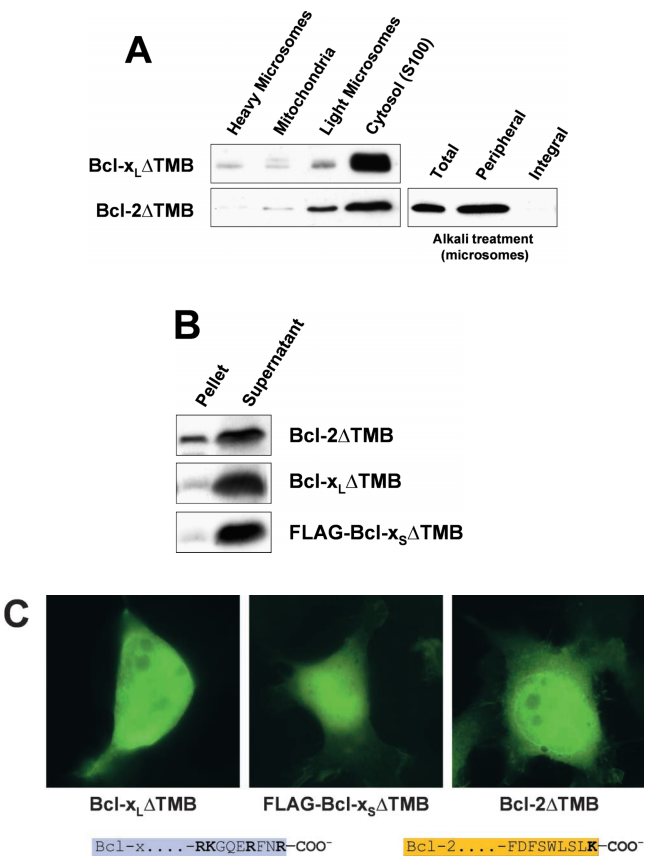

Figure 3. **Tailless Bcl-x$_L$ and Bcl-2 mutants are cytoplasmic and partially attached to light microsomes.** (A) Anti–Bcl-2 and anti–Bcl-x Western blots of subcellular fractions from HEK293 cells transiently transfected with Bcl-2 or Bcl-x$_L$ mutants lacking the last 21 amino acids (TMB domain) (Bcl-2ΔTMB and Bcl-x$_L$ΔTMB). In addition, a sodium carbonate (alkali) extraction of microsomes (as described in legend to Fig. 1 C) is shown for Bcl-2ΔTMB in the right panel. (B) Autoradiography of the IVTT products of Bcl-2ΔTMB, Bcl-x$_L$ΔTMB, and FLAG–Bcl-x$_S$ΔTMB, bound to microsomes (pellet) or remaining in the supernatant after spinning off the microsomes. (C) Anti–Bcl-2 and anti–Bcl-x immunofluorescence analysis of HeLa cells transiently overexpressing the three mutants.

These data suggest that the TMB region is crucial for effective membrane insertion of all three proteins.

The TMB of Bcl-x requires the preceding X domain for effective membrane insertion and mitochondrial targeting

To investigate whether the TMBs of Bcl-2 and Bcl-x$_L$/x$_S$ alone were sufficient for membrane targeting, they were separately fused to the COOH-terminal end of EGFP (Fig. 2 C). To our surprise, both the EGFP–TMB(Bcl-2) and EGFP–TMB(Bcl-x) proteins displayed a diffuse staining by whole cell fluorescence and mainly localized to a cytosolic fraction (Fig. 4, A and B), suggesting a role for additional amino acid sequences in Bcl-x and Bcl-2 for membrane targeting. The region preceding the TMBs, called the X domain, has no sequence homology between Bcl-2 and Bcl-x (Fig. 2 A) and thus could be responsible for the distinct subcellular targeting of the two proteins. Introduction of the X domain of Bcl-x into the EGFP–TMB(Bcl-x) construct (EGFP–X–TMB[Bcl-x]) or half of the X domain of Bcl-2 (amino acids WLSLK) into the EGFP–TMB(Bcl-2) construct (EGFP–X/2–TMB[Bcl-2]) (Fig. 2, A and C) gave identical targeting specificities as for wild-type Bcl-x$_L$/x$_S$ or Bcl-2, respectively (compare Fig. 4, A and B, to Fig. 1, A and E). The EGFP–X/2–TMB(Bcl-2) protein was immunodetected in all membrane fractions and produced a strong nuclear envelope/ER staining (Fig. 4, A and B). Adding the entire X domain and the preceding BH2 domain of Bcl-2 (EGFP–BH2X–TMB[Bcl-2]) did not alter its ubiquitous membrane distribution. By con-

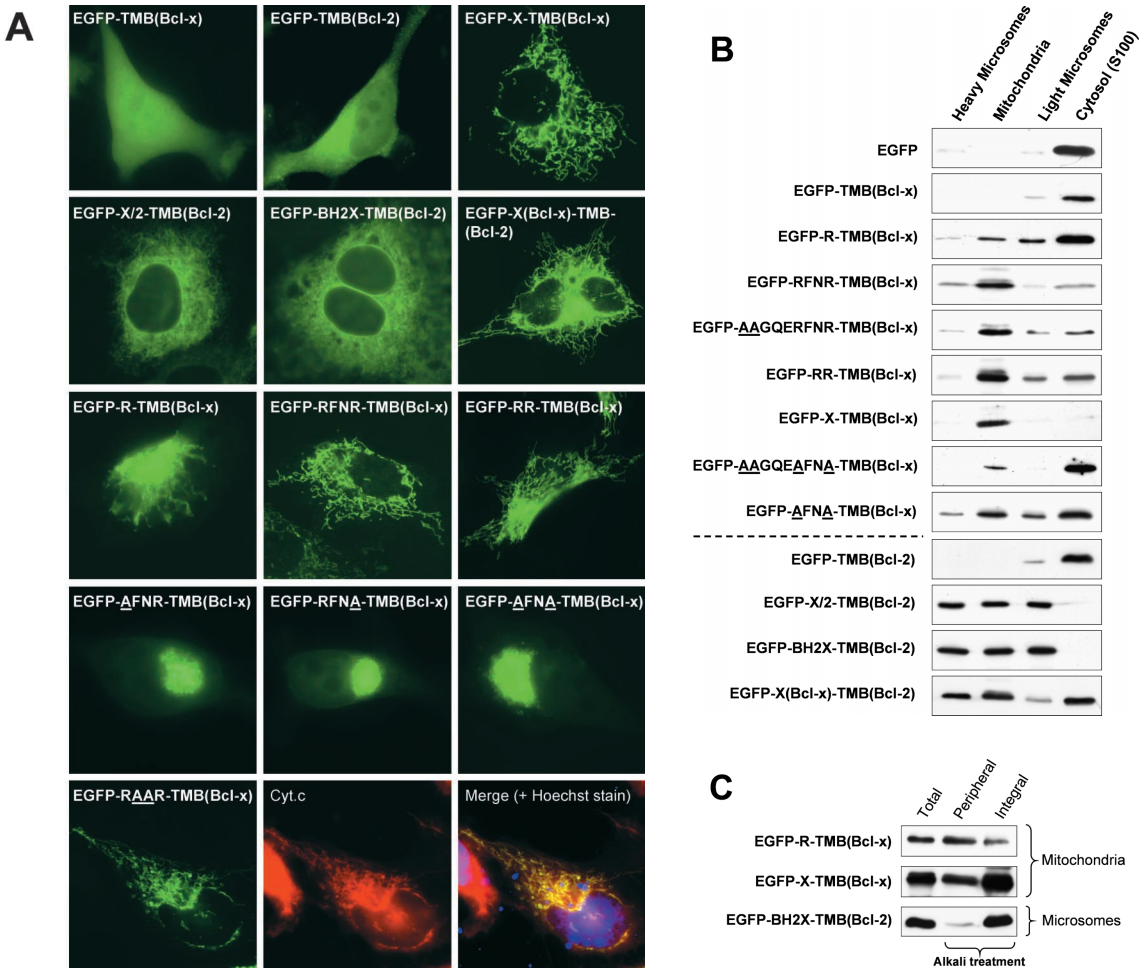

Figure 4. **Two basic amino acids of the X domain and the TMB of Bcl-x compose a signal that targets EGFP to mitochondria.** (A) Whole cell GFP fluorescence analysis of R6 cells transiently transfected with EGFP fused to various COOH-terminal mutants of Bcl-x and Bcl-2. The amino acid sequences of the mutants are described in Fig. 2 C. The mitochondrial colocalization marker is cytochrome c. Point mutations are underlined. (B) Anti-GFP Western blots of subcellular fractions from HEK293 cells expressing the various EGFP mutants. (C) Sodium carbonate extraction, as described in legend to Fig. 1 C, of mitochondria or microsomes isolated from HEK cells transfected with three selected EGFP mutants.

trast, the EGFP–X–TMB(Bcl-x) protein was only found in a mitochondrial fraction (Fig. 4 B) and was specifically targeted to elongated, mitochondrial structures (Fig. 4 A). Both the EGFP–BH2X–TMB(Bcl-2) and the EGFP–X–TMB(Bcl-x) proteins were difficult to extract from membranes by alkali treatment, indicating that the X domains directed the stable membrane insertion of the TMBs (Fig. 4 C). Consistent with a crucial role of the X domain of Bcl-x in mitochondrial targeting, Bcl-x_L lacking this domain (Bcl-$x_L\Delta$X) (Fig. 2 B) was found to reside majorly in the cytosol, despite the fact that it retained its TMB (Fig. 5 C). These data show that in both Bcl-2 and Bcl-x, the X domain cooperates with the TMB for membrane association and stable insertion. In Bcl-x, it additionally constitutes a mitochondrial sorting signal.

Switching the X-TMB region between Bcl-x and Bcl-2 alters membrane targeting selectivity

If the X-TMB region of Bcl-x is a bona fide mitochondrial targeting sequence that is absent in Bcl-2, then switching this region between the two molecules should alter their targeting properties. We assumed that these proteins did not undergo major conformational changes due to this exchange, as their three-dimensional structure is almost identical (Cory and Adams, 2002). As expected, a Bcl-2 mutant containing the X-TMB domain of Bcl-x (Bcl-2[X-TMB(Bcl-x)]; Fig. 2 B) was specifically targeted to mitochondria (Fig. 5, A and C). On the other hand, Bcl-x_L lost its specific mitochondrial association and localized to the cytoplasm, the nucleus, and other membranes upon acquisition of the X-TMB region of Bcl-2 (Bcl-x_L[X-TMB(Bcl-2)]; Fig. 2 B; Fig. 5 A; unpublished data). Thus, Bcl-2 can be specifically targeted to mitochondria in the presence of the X-TMB mitochondrial sorting signal from Bcl-x.

The TMB of Bcl-2 can only partially collaborate with the X domain of Bcl-x for mitochondrial sorting

Considering the possibility that the TMB of Bcl-2 may have mitochondrial sorting capacity when combined with the correct X domain, we generated a EGFP fusion protein that contained the X domain of Bcl-x and the TMB of Bcl-2. This EGFP–X(Bcl-x)–TMB(Bcl-2) fusion protein was better targeted to the mitochondrial fraction (Fig. 4 B) and lo-

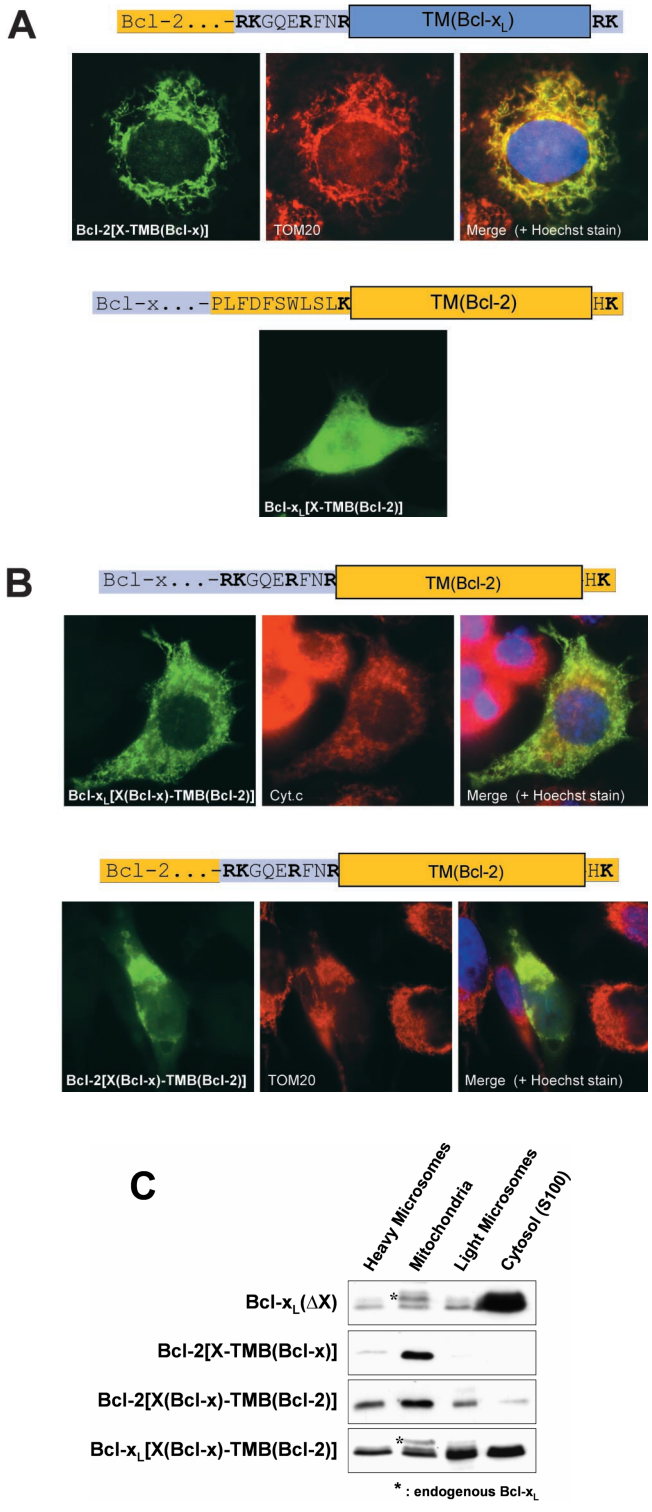

Figure 5. **Bcl-2 is specifically targeted to mitochondria upon acquisition of the X-TMB domain of Bcl-x.** (A and B) Anti–Bcl-2 and anti–Bcl-x immunofluorescence analysis of HeLa cells transiently transfected with Bcl-2 and Bcl-x_L chimera that had their X-TMB domains exchanged. The mitochondrial colocalization markers are cytochrome c or TOM20 as indicated. (C) Anti–Bcl-2 and anti–Bcl-x Western blots of subcellular fractions from HEK293 cells expressing the various chimeric mutants as well as a mutant of Bcl-x_L devoid of its X domain (Bcl-$x_L\Delta$X). An asterisk denotes the localization of endogenous Bcl-x_L.

calized more specifically to mitochondrial structures (Fig. 4 A) than the EGFP–X/2–TMB(Bcl-2) or EGFP–BH2X–TMB(Bcl-2) constructs (which contained the X domain of Bcl-2). However, the protein still appeared in heavy microsomes as well as in the cytosol (Fig. 4 B). Similar results were obtained when the X(Bcl-x)–TMB(Bcl-2) region was fused to Bcl-x_L (Bcl-x_L[X(Bcl-x)–TMB(Bcl-2)]) or to Bcl-2 (Bcl-2[X(Bcl-x)–TMB(Bcl-2)]). The chimeric proteins colocalized with mitochondrial markers (yellow in the merge) and were partially enriched in mitochondrial fractions, but significant amounts of the proteins resided in other cellular compartments (punctated green in the merge), such as on microsomes and in the cytoplasm (Fig. 5, B and C). These data suggest that although the X domain of Bcl-x can collaborate with the TMB of Bcl-2 for mitochondrial targeting, effective sorting requires the TMB of Bcl-x.

Two basic residues preceding the TMB of Bcl-x compose with the TMB a mitochondrial targeting signal, but additional basic residues in the X domain stabilize the signal

Which amino acids in the X domain of Bcl-x were crucial to cooperate with the TMB for mitochondrial targeting? Because this domain contains four basic residues (as compared with only one in Bcl-2) (Fig. 2 A), the role of these positive charges was investigated. First, the basic amino acid that lies closest to the TMB domain (Arg212) was introduced into the EGFP–TMB(Bcl-x) construct (Fig. 2 C). Compared with the EGFP–TMB(Bcl-x) protein, which was entirely cytosolic, EGFP–**R**–TMB(Bcl-x) partially localized to mitochondrial structures (Fig. 4 A) and was retained in a mitochondrial fraction (Fig. 4 B). However, most of the fusion protein still resided in the cytosol and on light microsomes (Fig. 4 B), and its membrane association was rather peripheral (Fig. 4 C). This is in agreement with the prominent cytoplasmic localization of the Bcl-$x_L\Delta$X mutant, which lacked the X domain but retained Arg212 and the TMB (Fig. 2 B; Fig. 5 C). Thus, Arg212 was insufficient for specific mitochondrial targeting and membrane insertion. The preceding region was then extended to the next basic residue (Arg209) (Fig. 2, A and C). The respective EGFP–**R**FN**R**–TMB(Bcl-x) fusion protein displayed a striking mitochondrial localization pattern (Fig. 4 A) and predominantly resided in mitochondrial membranes in a stably inserted form (Fig. 4 B; unpublished data). Despite that, a low amount of the protein remained in cytosolic and microsomal fractions when compared with EGFP–X–TMB(Bcl-x) (Fig. 4 B) or Bcl-x_L (Fig. 1 A). Additional amino acids in the X region, in particular the first two basic residues (Arg204 and Lys205), were studied for their capacity to assist mitochondrial targeting (Fig. 2 A). As compared with EGFP–X–TMB(Bcl-x), which was entirely mitochondrial, the EGFP–AAGQE**R**FN**R**–TMB(Bcl-x), where Arg204 and Lys205 were mutated to Ala, similarly appeared in microsomes and the cytosol as EGFP–**R**FN**R**–TMB(Bcl-x) (Fig. 4 B). These results showed that Arg204 and Lys205 of the X domain slightly increased the mitochondrial sorting property of **R**FN**R**–TMB(Bcl-x). Consistent with this notion, an EGFP–X–TMB(Bcl-x) fusion protein

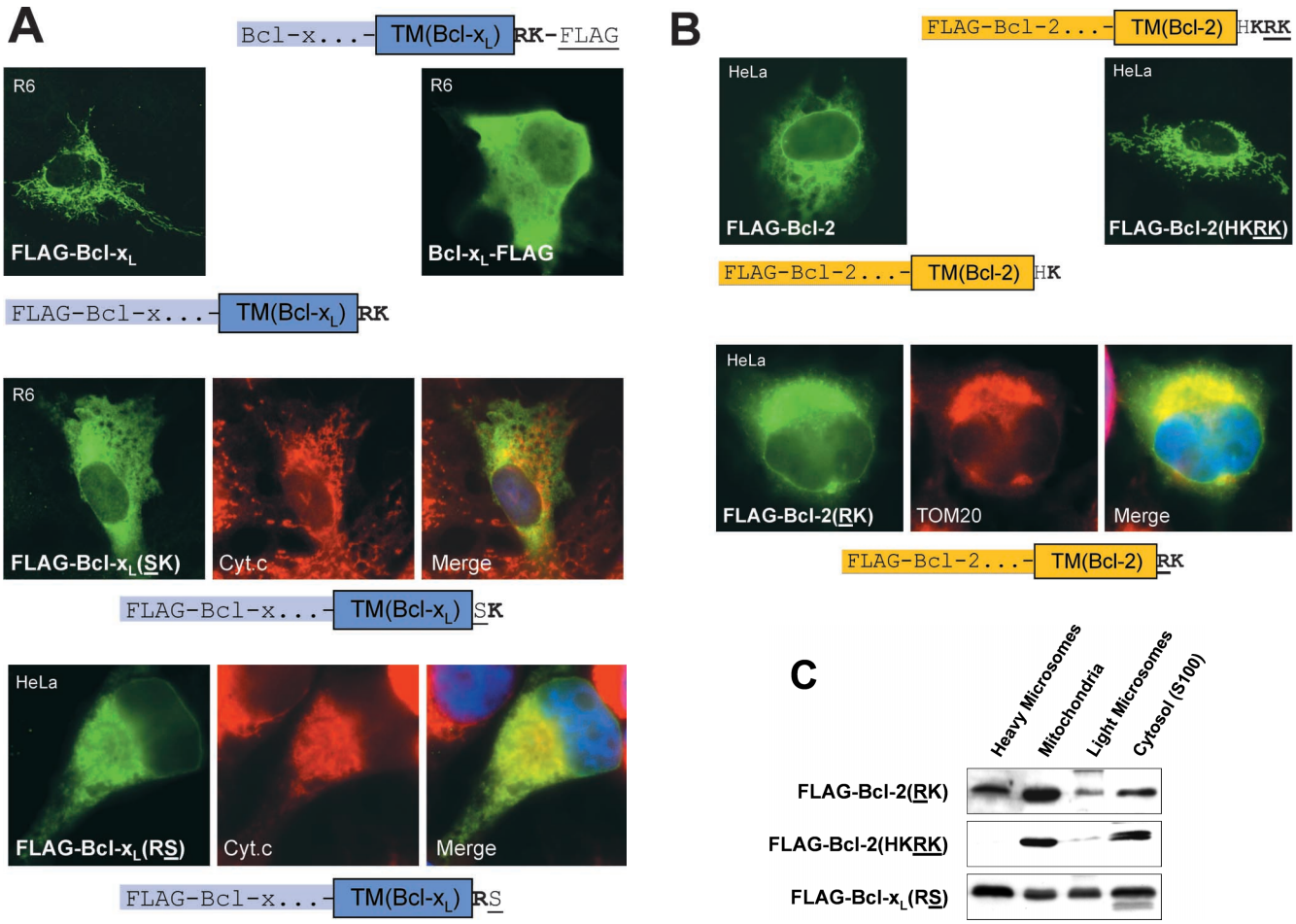

Figure 6. **MOM targeting also requires two basic residues downstream of the TM of Bcl-x and Bcl-2 accumulates on mitochondria by increasing the basicity surrounding its TM.** (A and B) Anti–Bcl-2 and anti–Bcl-x immunofluorescence analysis of HeLa or R6 cells (as indicated) transiently transfected with FLAG-tagged Bcl-x_L mutants that had their COOH-terminal basic residues exchanged to serines (A) or FLAG-tagged Bcl-2 mutants that contained additional basic residues at their COOH termini (B). In addition, the localization of COOH-terminal FLAG-tagged Bcl-x_L is shown (Bcl-x_L–FLAG). The mitochondrial colocalization markers are cytochrome c or TOM20 as indicated. (C) Anti-FLAG Western blots of subcellular fractions from HEK293 cells expressing the mutants described under A and B.

that had all basic residues exchanged to alanine (EGFP–AAGQEAFNA–TMB[Bcl-x]) lost most of its mitochondrial targeting function and became largely cytosolic (Fig. 4 B). In summary, although the **R**FN**R** sequence is both required and sufficient to cooperate with the TMB for mitochondrial targeting, Arg204 and Lys205 of the X domain slightly enhance this targeting.

To prove that the two Arg, and not the Phe/Asn sequence, in **R**FN**R** conferred the mitochondrial signaling, we mutated these amino acids (Fig. 2 C). EGFP fusion proteins with both Phe and Asn deleted (EGFP–**RR**–TMB[Bcl-x]) or exchanged to Ala (EGFP–**R**AA**R**–TMB[Bcl-x]) localized to mitochondria with a similar efficiency as the **R**FN**R** construct (Fig. 4, A and B). By contrast, EGFP fusions that had one or both Arg exchanged to Ala (EGFP–AFN**R**–TMB[Bcl-x], EGFP–**R**FNA–TMB[Bcl-x], and EGFP–AFNA–TMB[Bcl-x]) lost most of their mitochondrial association and became cytosolic and microsomal (Fig. 4, A and B; unpublished data). These mutants also showed a cytoplasmic clustering (Fig. 4 A) that was lethal to cells 48 h after transfection (unpublished data). Thus, the two basic residues, and not the amino acids between these residues, cooperate with the TMB of Bcl-x for mitochondrial sorting.

The mitochondrial sorting signal of Bcl-x also requires at least two basic residues downstream of the TM

It was striking that the EGFP–AAGQEAFNA–TMB(Bcl-x) mutant still specifically associated with mitochondria whereas the EGFP–TMB(Bcl-x) mutant was fully cytosolic (Fig. 4 B). This suggested that the TMB of Bcl-x had a partial mitochondrial targeting capacity even though the preceding X domain did not contain the flanking basic amino acids. The TMB consists of the hydrophobic TM domain followed by two basic amino acids in the B domain (**RK**) (Fig. 2 A). To investigate whether these basic amino acids played a role in mitochondrial targeting, we mutated them in a FLAG-tagged version of Bcl-x_L (to distinguish the mutants from endogenous Bcl-x_L) (Fig. 2 B). FLAG-tagged Bcl-x_L specifically localized to mitochondria like its nontagged counterpart (Fig. 6 A). By contrast, eliminating one positive

charge of the B domain by mutating Arg232 or Lys233 to Ser was sufficient to prevent the specific mitochondrial association of Bcl-x_L. Both the FLAG–Bcl-x_L(S**K**) and FLAG–Bcl-x_L(**R**S) mutants lost most of the mitochondria-specific localization pattern, stained the nuclear envelope and the ER membrane (Fig. 6 A), and resided on all intracellular membranes and in the cytosol in a similar way as Bcl-2 (Fig. 6 C). Moreover, the addition of an eight–amino acid FLAG peptide to the COOH terminus of Bcl-x_L (Bcl-x_L–FLAG; Fig. 2 B) led to a cytosolic distribution of Bcl-x_L (Fig. 6 A), perhaps by neutralizing the extreme COOH-terminal RK amino acids. These data show that the mitochondrial sorting signal of Bcl-x does not only require two basic residues upstream (in the X domain) but also at least two basic residues downstream of its TM.

Bcl-2 can be specifically sorted to mitochondria by increasing COOH-terminal basicity

Based on the Bcl-x results, we reevaluated the mitochondrial targeting properties of the TMB of Bcl-2. The two COOH-terminal amino acids in the B domain of Bcl-2 are His-Lys (H**K**) instead of Arg-Lys (**RK**) (Fig. 2 A). Although His is a basic amino acid, its basicity is weak, and this could be responsible for the lack of mitochondrial targeting. To investigate this possibility, we mutated His238 to Arg in FLAG-tagged Bcl-2 (FLAG-Bcl-2[**R**K]) (Fig. 2 B). As shown in Fig. 6 B, FLAG–Bcl-2 stained the nuclear envelope and the associated ER like Bcl-2. This was also the case for FLAG–Bcl-2(**R**K) (Fig. 6 B) but the mutant was already more abundant in a mitochondrial fraction (compare Fig. 6 C with Fig. 1 A). We therefore increased the basicity of the COOH terminus of Bcl-2 by one more basic amino acid (H**KRK**) (FLAG-Bcl-2[H**KRK**]) (Fig. 2 B). This mutant was concentrated in a mitochondrial fraction (Fig. 6 C) and nicely stained mitochondria in both HeLa (Fig. 6 B) and R6 cells (unpublished data), although some cytosolic localization remained. These data indicate that the TM region of Bcl-2 can contribute to mitochondrial targeting, provided that it is surrounded by sufficient basicity.

Discussion

In this study, a novel COOH-terminal signal sequence for the specific targeting and stable insertion of Bcl-x_L and Bcl-x_S to the MOM was identified. Bcl-2 lacks this signal and therefore stably inserts into several intracellular membranes in a relatively nonspecific manner. This subcellular distribution was seen to occur for endogenous and overexpressed Bcl-x_L/x_S and Bcl-2 proteins in at least three different cell lines by two different techniques.

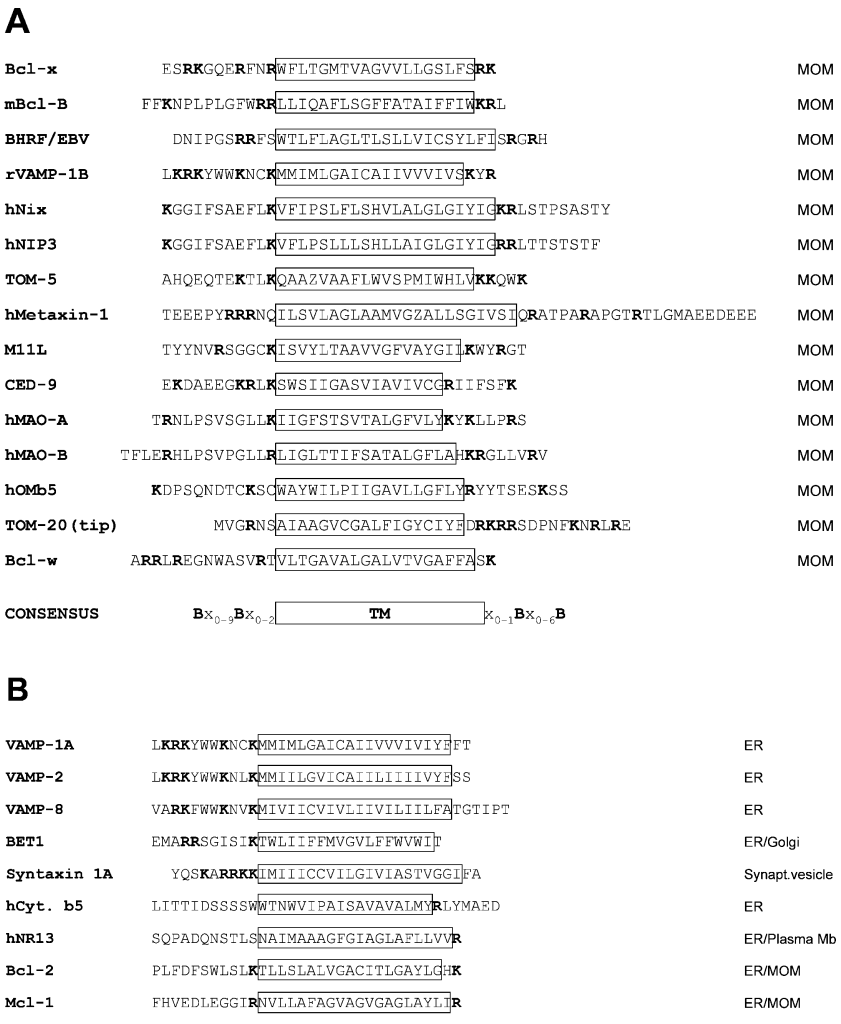

Figure 7. **Schematic representation of MOM- and ER-targeted proteins.** (A) Amino acid sequences of MOM-targeted proteins whose COOH termini conform to the possible **B**x_{0-9}**B**x_{0-2}-TM-x_{0-1}**B**x_{0-6}**B** consensus sequence or contain a high basicity at one end of their TM-like FLAG–Bcl-2(H**KRK**) (TOM20 and Bcl-w). (B) Amino acid sequences of proteins that contain only one basic residues at each end (Bcl-2 and Mcl-1), or basic residues at only one end of the TM and thus localize to extra-mitochondrial sites.

Recent studies have shown that the targeting of tail- or tip-anchored proteins to the MOM depends on the length and hydrophobicity of a TM domain of 17–23 hydrophobic amino acids and a short hydrophilic sequence rich in basic residues either before or after the TM domain (McBride et al., 1992; Kuroda et al., 1998; Egan et al., 1999; Kanaji et al., 2000; Mihara, 2000; Horie et al., 2002). We however noted that these proteins contained basic amino acids at both ends of their TM (Fig. 7 A). Although a targeting role of positive charges on both sides have recently been suggested for the M11L protein of myxoma virus and, astonishingly, for Bcl-2, no mutagenesis analysis was performed to support this hypothesis (Everett et al., 2000). Here we combined both whole cell fluorescence and immunoblot analysis of pure organelle fractions to show that effective targeting of Bcl-x to the MOM requires at least two basic residues at both ends of the TM domain. Neither the hydrophobicity nor the length of the TM domain appear to play any role in targeting, as the TMs of Bcl-2 and Bcl-x have a similar degree of hydrophobicity and length despite different subcellular localizations. It may however be important at which distance the basic residues are located with respect to the TM domain, although this has not yet been directly tested. By comparing the MOM targeting sequences of several proteins, we propose that one basic residue is within one or two amino acids of the COOH and NH_2 termini of the TM domain, respectively, whereas the second basic residue is up to six or nine amino acids distant from the first (Fig. 7 A). A comparison of the subcellular localization of the EGFP–**R**FN**R**–TMB(Bcl-x), EGFP–**R**AA**R**–TMB(Bcl-x), and EGFP–**RR**–TMB(Bcl-x) constructs (Fig. 4) further suggests that in the case of Bcl-x, the nature and number of the amino acids between the basic residues do not play a mitochondrial targeting role. On the basis of these data, a new putative consensus sequence for MOM signaling/anchoring is proposed in the form of **B**x_{0-9}**B**x_{0-2}-TM-x_{0-1}**B**x_{0-6}**B** (where **B** stands for basic residues and x for any amino acid). This consensus is fulfilled by numerous proteins that are tail anchored in the MOM, such as the Bcl-2 family members Bcl-x, Bcl-B, BHRF-EBV, Nip3, Nix, and CED-9 as well as VAMP-1B, TOM-5, metaxin-1, myxoma viral M11L, monoamine oxidase A and B, and mitochondrial cytochrome b5 (Fig. 7 A). In the case of TOM20 and the Bcl-2 family member Bcl-w, which are mitochondrial proteins but display only one positive charge at one end of the TM, the other side contains a higher basicity (three to four basic residues) (Fig. 7 A), as seen with the mitochondria-targeted FLAG–Bcl-2(H**KRK**) construct (Fig. 6, B and C). By contrast, a variety of proteins that are targeted to the ER and other intracellular membranes lack the MOM targeting sequence, either because they contain basic residues at only one end of the TM (VAMP-1A, VAMP-2, VAMP-8, BET1, syntaxin 1A, microsomal cytochrome b5, and Bcl-2 homologue NR13) or do not have sufficient basicity at both ends (Bcl-2 and its homologue Mcl-1) (Fig. 7 B). Thus, the consensus sequence proposed here is more predictive for MOM sorting than the sequences previously reported. Exceptions are the pro-apoptotic Bcl-2 family members Bax and Bak, which contain basic residues only at one end of the TM but nevertheless localize to the MOM. These proteins probably require additional cellular factors to unleash the COOH-terminal mitochondrial targeting signal, which is folded back into the molecule after synthesis (Suzuki et al., 2000; unpublished data).

How does this signal sequence find the MOM and tail anchor the protein in this membrane? A multistep mechanism has been proposed in which the MOM-targeted protein first associates with cytosolic chaperones during or shortly after translation (Millar and Shore, 1996). The function of the chaperones is possibly to shield the emergent hydrophobic COOH terminus from the cytosol until insertion into the target membrane can be achieved (Egan et al., 1999; Beddoe and Lithgow, 2002). The chaperone-bound protein may then be transferred to a hypothetical receptor protein on the mitochondrial surface. Finally, the receptor-bound protein is passed to an insertion complex that accomplishes integration of the protein into the bilayer. It is unclear at present whether proteins targeted to the MOM by COOH-terminal signal/anchors utilize the same import machinery as matrix-directed proteins (TOM proteins), although there are some suggestions that at least one of these protein components is employed (Millar and Shore, 1996). Although tip-anchored proteins, such as TOM20, insert into the MOM independent of import receptors and then assemble into the TOM complex (Schneider et al., 1991), it is possible that tail-anchored proteins, such as Bcl-x_L and Bcl-x_S, require specific receptors. Analysis of the tail-anchored VAMP-1B protein revealed that association of this protein with the MOM is saturable, which suggests the existence of a receptor (Lan et al., 2000). In this study, we detected increasing amounts of Bcl-x_L in the cytosol or supernatant when the protein was gradually overexpressed in HeLa or R6 cells or given to isolated mitochondria after in vitro translation. Although this may indicate the presence of a receptor, trypsin stripping experiments of the mitochondrial surface have not yet supported this view, at least not for a protein receptor (unpublished data). As Bcl-x_L and Bcl-x_s become stably inserted in the lipid bilayer after targeting (Fig. 1, C and D), we propose that their interactions with putative receptors are only transient. This may also account for the interaction of Bcl-2 with TOM20 in yeast, as recently reported by Motz et al. (2002), although our data do not support such an interaction as a major determinant in the subcellular localization of Bcl-2 in mammalian cells. We can also exclude that these mitochondrial receptors are other members of the Bcl-2 family, as interactions between these proteins occur via the hydrophobic groove (BH1/BH2 domain) or the BH3 domain rather than the COOH terminus (Chittenden et al., 1995; Zha et al., 1996; Sattler et al., 1997).

Kanaji et al. (2000) reported that the TM region of TOM20 functions by itself as a signal-anchor sequence to target GFP fusions to the ER. Bcl-2 and Bcl-x_L both have a TM with similar hydophobicity as TOM20. Surprisingly, however, these hydrophobic TM domains were insufficient for membrane targeting and insertion. Even the addition of a basic residue in front of the TMB (EGFP–**K**–TMB[Bcl-2], data not shown; or EGFP–**R**–TMB[Bcl-x], Fig. 4, A and B) did not majorly enhance membrane targeting, and the small amount of protein that was membrane associated was loosely attached. Only when the sequence included the targeting motif, i.e., either the two basic residues NH_2-terminal to the TMB of Bcl-x_L or the X domain ahead of the TMB of Bcl-2,

were the respective proteins stably inserted into membranes. This supports the notion that targeting and anchoring are intimately linked, i.e., the same sequence functions as a targeting and insertion device (for review see Mihara, 2000).

Quantitative immunoelectron microscopy revealed that, depending on the cell type, 55–85% of Bcl-2 molecules are localized to the ER, and the remainder are inserted into the MOM of mammalian cells (Krajewski et al., 1993; Lithgow et al., 1994). In yeast, a fusion protein between the last 33 amino acids of Bcl-2 and a reporter protein is even exclusively targeted to the ER (Egan et al., 1999). We were unable to find any sequences in Bcl-2 that would target this protein specifically to any membrane in rat and human cells, thus excluding the possibility that this protein contains multiple targeting signals, as had been suggested (Lan et al., 2000). Moreover, in contrast to other ER-bound proteins such as VAMP-1A, the binding of Bcl-2 and microsomal cytochrome b5 to ER microsomes has been reported to be spontaneous and nonsaturable (Kim et al., 1997). This strongly suggests that Bcl-2 may not be targeted to this membrane but distributes on all intracellular membranes depending on the kinetic preference for one membrane over another (Wattenberg and Lithgow, 2001). Nuclear outer membrane and ER staining of Bcl-2 by immunofluorescence (Fig. 1 E; Fig. 4 A) may therefore simply reflect the fact that this endomembrane system has the largest surface in the cell. It was noticed that the two basic amino acids located after the TM domain of Bcl-2, His238-Lys239, may not be sufficient for MOM targeting, and that some portion of the protein may leak out of the mitochondrial transport apparatus, allowing Bcl-2 to be transported to or associated with the ER or other membranes (Kuroda et al., 1998). We show here that this potential "leaking out" can be prevented by increasing the basicity at the COOH terminus. Thus, the COOH terminus of Bcl-2 can be converted into a signal for the MOM, but usually acts as a nontargeting device in the wild-type protein.

An obvious question is whether Bcl-2 and Bcl-x_L exert different functions depending on their intracellular site of localization. When Bcl-2 was specifically targeted to mitochondria (e.g., Bcl-2[X-TMB(Bcl-x)]) and tested for its ability to block staurosporine- or brefeldin A/cycloheximide-induced apoptosis of HeLa cells, it was found that the anti-apoptotic activity of the mutant was only slightly reduced when compared with the wild-type forms (unpublished data). Similarly, Bcl-x_L targeted to the ER via the tail of the microsomal cytochrome b5 (unpublished data), or Bcl-x_L carrying the COOH terminus of Bcl-2 (Bcl-x_L[X-TMB (Bcl-2)]), exhibited a survival activity that was nearly as efficient as that of the wild-type Bcl-x_L. Also, consistent with previous results (Borner et al., 1994), cytoplasmic mutants of Bcl-2 and Bcl-x_L still possess partial anti-apoptotic activity although this continued activity may be due to a minor association between the tailless mutants and membranes, as shown in Fig. 3. Thus, our data support the notion that Bcl-2 and Bcl-x_L are exchangeable survival factors that, independent of their subcellular localization, repress a common cell death pathway (Chao et al., 1995). Whereas previous reports have proposed nonredundant functions of Bcl-2 and Bcl-x_L (Gottschalk et al., 1994; Coulson et al., 1999; Yuste et al., 2002), these studies were all undertaken using cells that overexpressed these proteins to high levels. Here we have begun to study the subcellular localization of the endogenous proteins. It will now be crucial to examine whether endogenous Bcl-x_L exerts a mitochondria-specific function that cannot be replaced by Bcl-2, or whether endogenous Bcl-2 regulates ER-associated events of apoptosis that are not affected by Bcl-x_L. Such organelle-specific actions may include post-translational modifications such as the recently published deamidation of Bcl-x_L, which inactivates the endogenous protein in response to DNA damaging agents and does not occur on Bcl-2 (Deverman et al., 2002). Studies using Bcl-x and Bcl-2 knockout mice have further shown that these proteins play nonredundant roles in tissues and cells (Veis et al., 1993; Motoyama et al., 1995). Although this may simply reflect a difference in tissue distribution, it could also be a consequence of distinct subcellular localizations and/or molecular mechanisms to oppose cell death of Bcl-2 and Bcl-x_L. To solve this issue, one would for example need to express Bcl-2 on mitochondria at similar levels as Bcl-x_L in a Bcl-x–null tissue and examine whether this now rescues the knockout phenotype. Irrespective of the result, the data presented here improve our understanding of how proteins are specifically targeted to the MOM and how they differ from proteins that accumulate on other intracellular membranes.

Materials and methods

cDNAs and site-directed mutagenesis

The cDNAs for human and mouse Bcl-2, human Bax, FLAG-tagged human Bcl-2, and Bcl-x_S and FLAG-tagged Bcl-x_S devoid of the last 21 amino acids (FLAG–Bcl-$x_S\Delta$TMB) were generated and subcloned into the pcDNA3 or pcDNAampI vectors (Invitrogen) as previously described (Borner et al., 1994; Conus et al., 2000a; Lindenboim et al., 2000). The cDNA for human Bcl-x_L was obtained from G. Nunez (University of Michigan, Ann Arbor, MI) (in pcDNA3). pEF-Bcl-x_L–FLAG-puro was a gift from D. Huang (Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) (Morishi et al., 1999). All EGFP constructs were made in the pEGFP-C1, -C2, or -C3 vectors (Invitrogen) except for TAP(I-VI)–EGFP (EGFP-N3 vector; obtained from J. Neefjes, Netherlands Cancer Institute, Amsterdam, Netherlands) (Vos et al., 2000). The cDNAs for the various (FLAG–) Bcl-2 and Bcl-x_L mutants, the Bcl-2/Bcl-x_L chimeras, and the EGFP fusion proteins were generated by PCR as described in the online supplemental material (available at http://www.jcb.org/cgi/content/full/jcb.200210084/DC1). All constructs were verified by dideoxynucleotide sequencing.

Protein expression and subcellular fractionation

Human embryonic kidney cells (HEK293) were grown on 100 mm until ~80% confluent and then transfected with 10 μg of plasmid DNA using 25 μl of Superfect (QIAGEN) as described by the manufacturer. After 3–6 h, the Superfect–DNA complexes were removed and the cells were cultured for another 42 h. 3–5 × 10^7 HEK293 cells were homogenized in MSH buffer (210 mM mannitol, 70 mM sucrose, 20 mM Hepes, 1 mM EDTA, pH 7.4) plus protease inhibitors, and nuclei and cellular debris were removed by centrifugation at 500 *g* for 5 min. The post-nuclear supernatant was centrifuged at 5,100 *g* for 10 min to obtain the crude mitochondrial pellet. For further purification, the crude mitochondria were laid on top of a 1–2 M linear sucrose gradient and centrifuged at 52,000 *g* for 90 min in a SW41 rotor (Beckman Coulter). Purified mitochondria, which banded at a region corresponding to 1.4 M sucrose, were carefully collected and diluted to 0.25 M sucrose before centrifugation at 30,000 *g* for 30 min. The resulting pellets were solubilized in buffer H (20 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 6 mM β-mercaptoethanol, pH 7.5) containing 1% SDS. The band at 1.0 M sucrose, which corresponded to heavy microsomes, was similarly processed. Centrifugation of the post-mitochondrial supernatant at 100,000 *g* for 60 min yielded the light microsomal pellet and the cytosolic supernatant. Cytosols were immediately frozen; light microsomes were resuspended in buffer H containing 1% SDS. All frac-

tions were tested for their purity by immunoblot analysis using antibodies against organelle-specific proteins.

Submitochondrial fractionation

Fractionation of mitochondria isolated from rat liver or HEK293 cells into inner membrane, outer membrane, and matrix components was performed exactly as previously described (Hoppel et al., 1998). Equal amounts of protein were loaded on SDS gels for anti–Bcl-x immunoblot analysis.

Sodium carbonate extractions

Crude mitochondria or microsomes (as prepared above) were resuspended in 0.1 M sodium carbonate (pH 12) and incubated for 20 min on ice. After centrifugation, the supernatant (containing the alkali-extractable proteins) was titrated to neutral pH with HCl. The pellet (containing the alkali-resistant fraction) was washed three times and then resuspended in buffer H (see above) containing 1% SDS. About 10% of the crude mitochondria or microsomal fraction was directly solubilized in buffer H containing 1% SDS (detergent control).

SDS-PAGE and Western blotting

30 μg of protein from subcellular fractions and sodium carbonate extractions were run on 15% SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membrane (BDH), and immunodetected by anti-h/m/rBcl-x (S-18, 1:2,000; Santa Cruz Biotechnology, Inc.), anti–hBcl-2 (clone 124, 1:1,000; DakoCytomation), anti–mBcl-2 (10C4, 1:1000; Zymed Laboratories), anti-GFP/JL-8 (Living colors®, 1:1,000; Invitrogen), anti-KDEL (1:1,000; StressGen Biotechnologies), anti-grp78/Bip (1:1,000; StressGen Biotechnologies), and anti-COX/VIc (1:300; Molecular Probes) primary antibodies followed by peroxidase-coupled, F_c-specific goat anti–rabbit or anti–mouse secondary antibodies (Sigma-Aldrich). Immunodetection was performed by ECL (Pierce Chemical Co.). Equal protein loading was confirmed by amido black staining of the membrane.

Immunofluorescence analysis

Rat embryo fibroblasts (R6) or human epithelioid cervical carcinoma (HeLa) cells were grown on 12-mm glass coverslips and then were transiently transfected with 0.8 μg of plasmid DNA and 3.2 μl of Superfect as described above. At 24 h after transfection, cells were fixed in 4% paraformaldehyde and permeabilized with 0.05% saponin and ice cold acetone. The cells were incubated with anti-h/m/rBcl-xL (S-18, 1:150; Santa Cruz Biotechnology, Inc.), anti–hBcl-2 (clone 124, 1:100, DakoCytomation), or anti–mBcl-2 (10C4, 1:100; Zymed Laboratories) in the presence of either anti–cytochrome c (1:50; BD Biosciences), anti–TOM-20 (1:300; gift from B. Hanson, Institute of Molecular and Cell Biology, Singapore), or anti-calnexin (1:100; StressGen Biotechnologies) as colocalization markers for 90 min followed by fluorescein- and/or Texas red–conjugated goat anti–rabbit or anti–mouse secondary antibodies (Jackson ImmunoResearch Laboratories) for another 60 min. After postfixation in 4% paraformaldehyde containing 2 μg/ml Hoechst 33342 dye (Molecular Probes), the anti-fading agent Slowfade (Molecular Probes) was added, and the cells were viewed under a ZEISS Axioplan fluorescence microscope using standard filter for green and red fluorescence. Pictures were taken with a ZEISS digital camera and processed with the ZEISS AxioVision 3.06 software.

In vitro transcription–translation (IVTT)

The TNT Quick T7-coupled reticulocyte lysate system (Promega) was used essentially as described by the manufacturer. In vitro transcription–translation (IVTT) was performed at 30°C for 60 min in the presence of 0.6 mCi/ml [^{35}S]methionine (Amersham Biosciences) in a volume of 25 μl. The reaction was stopped by the addition of 10 μg/ml cycloheximide (CHX). The lysate was centrifuged for 15 min at 12,000 *g* to remove possible nonsoluble aggregates. For membrane association–insertion assays, freshly prepared crude mitochondria and microsomes from HEK293 cells were resuspended in MSH buffer (pH 7.5). 10 μl of the precleared IVTT product was mixed with 25 μl of membranes and incubated at 30°C for 20 min. After a centrifugation step (12,000 *g* for mitochondria, 100,000 *g* for microsomes), supernatants were collected and the membranes were washed three times in MSH buffer before analysis by SDS-PAGE and detection by autoradiography. For alkaline extraction of mitochondria/microsomes carrying IVVT products, the membranes were treated with sodium carbonate as described above.

Online supplemental material

Details about the generation of the various Bcl-2, Bcl-x_L, and EGFP mutants by PCR can be found a http://www.jcb.org/cgi/content/full/jcb.200210084/DC1.

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