Reversible Membrane Interaction of BAD Requires two C-terminal Lipid Binding Domains in Conjunction with 14-3-3 Protein Binding

Received for publication, January 11, 2006, and in revised form, March 31, 2006 Published, JBC Papers in Press, April 7, 2006, DOI 10.1074/jbc.M600292200

Mirko Hekman†, Stefan Albert†, Antoine Galmiche†, Ulrike E. E. Rennefahrt†, Jochen Fueller‡, Andreas Fischer‡, Dirk Puehringer‡, Stefan Wiese§, and Ulf R. Rapp†‡

From the †Institute for Medical Radiation and Cell Research and §Institute for Clinical Neurobiology, University of Wuerzburg, 97078 Wuerzburg, Germany

BAD is a Bcl-2 homology domain 3 (BH3)-only proapoptotic member of the Bcl-2 protein family that is regulated by phosphorylation in response to survival factors. Binding of BAD to mitochondria is thought to be exclusively mediated by its BH3 domain. We show here that BAD binds to lipids with high affinities, predominantly to negatively charged phospholipids, such as phosphatidylserine, phosphatidic acid, and cardiolipin, as well as to cholesterol-rich liposomes. Two lipid binding domains (LBD1 and LBD2) with different binding preferences were identified, both located in the C-terminal part of the BAD protein. BAD facilitates membrane translocation of Bcl-X<sub>L</sub> in a process that requires LBD2. Integrity of LBD1 and LBD2 is also required for proapoptotic activity in vivo. Phosphorylation of BAD does not affect membrane binding but renders BAD susceptible to membrane extraction by 14-3-3 proteins. BAD can be removed efficiently by 14-3-3-ζ, η, and less efficiently by other 14-3-3 isoforms. The assembled BAD-14-3-3 complex exhibited high affinity for cholesterol-rich liposomes but low affinity for mitochondrial membranes. We conclude that BAD is a membrane-associated protein that has the hallmarks of a receptor rather than a ligand. Lipid binding is essential for the proapoptotic function of BAD in vivo. The data support a model in which BAD shuttles in a phosphorylation-dependent manner between mitochondria and other membranes and where 14-3-3 is a key regulator of this relocation. The dynamic interaction of BAD with membranes is tied to activation and membrane translocation of Bcl-X<sub>L</sub>.

Programmed cell death is a conserved pathway essential for all multicellular organisms. Many growth factors and cytokines, such as interleukin-3, function as survival factors that suppress apoptosis. Proteins of the Bcl-2 family represent crucial players in the regulation of apoptosis. Whereas some members prevent cell death (e.g. Bcl-2 and Bcl-X<sub>L</sub>), others exhibit proapoptotic activities (e.g. Bax and Bak). Bcl-2 family members are characterized by conserved domains designated Bcl-2 homology domains 1–4 (BH1–BH4). These domains adopt α-helical secondary structures (for a review, see Ref. 1). The balance between pro- and antiapoptotic proteins can be influenced by a third group of proteins termed BH3-only proteins (e.g. Bik,Bid,Bim,Puma,BAD, and Noxa).

BAD promotes apoptosis by forming heterodimers with the survival proteins Bcl-2 and Bcl-X<sub>L</sub>. Complex formation prevents Bcl-2/Bcl-X<sub>L</sub> from binding with Bax (2) or “Bax activators” of the BH3 only subfamily of Bcl-2, such as Bid and Bim (3–5). Dephosphorylated BAD in complex with Bcl-X<sub>L</sub> is found at the outer mitochondrial membrane (6), and phosphorylation of specific serine residues (Ser-112 and Ser-136) results in cytoplasmic sequestration by 14-3-3 proteins (7). Phosphorylation of BAD at serine 155 disrupts the association of BAD with Bcl-2 and Bcl-X<sub>L</sub> and promotes cell survival (8). Thus, the phosphorylation status of BAD on these serines reflects one of the key checkpoints for cell death or survival. Published data suggest that RAF kinases (9–12), protein kinase A (13), Akt/protein kinase B (14), and PAK (15) link growth factor receptors to phosphorylation of BAD and cell survival. The interaction of survival kinases such as RAF and Akt with BAD requires association with Hsp70 and Bag-1 at the outer mitochondrial membrane (10, 12, 16). In fact, in the absence of Bag-1 there is a marked reduction of Akt and RAF at mitochondria and Ser-136 of BAD remains specifically unphosphorylated. Presumably, the BAD phosphorylation complex is not properly assembled in the absence of Bag-1 (16).

Most members of the Bcl-2 family contain a C-terminal hydrophobic transmembrane domain, indicating that these proteins may exist as integral membrane proteins. The lipophilic properties of the C-terminal domains of some pro- and antiapoptotic proteins (Bax, Bak, Bcl-X<sub>L</sub>, and Bcl-2) have been investigated recently (17–19). Kuwana <i>et al.</i> (20) reported that Bid and Bax require interactions with specific mitochondrial lipids, such as cardiolipin, for their proapoptotic effects. Since the three-dimensional structure of BAD is not known and the interactions of BAD with lipids have not been reported, we investigated in this study the possible associations of BAD with membranes (21). Complex lipid compositions resembling plasma membranes, cholesterol-rich domains, and mitochondrial membranes were employed. We report here for the first time that BAD exhibits pronounced lipophilic properties, associating with negatively charged phospholipids and cholesterol-rich liposomes. Based on both in vivo and in vitro data, we postulate a regul-

---

*This work was supported by Deutsche Forschungsgemeinschaft Grants SFB 487, SFB 581, and RA 642/11 and the Scheel Foundation (Priority Program on Apoptosis). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S5.

§1 These two authors contributed equally to this work.

‡2 To whom correspondence should be addressed: University of Wuerzburg, Institute for Medical Radiation and Cell Research, Versbacher Str. 5, 97078 Wuerzburg, Germany. Tel.: 49-931-201-45141; Fax: 49-931-201-45835; E-mail: rappur@mail.uni-wuerzburg.de.

‡1 These two authors contributed equally to this work.

‡2 To whom correspondence should be addressed: University of Wuerzburg, Institute for Medical Radiation and Cell Research, Versbacher Str. 5, 97078 Wuerzburg, Germany. Tel.: 49-931-201-45141; Fax: 49-931-201-45835; E-mail: rappur@mail.uni-wuerzburg.de.

© 2006 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
**BAD Association with Liposomes and 14-3-3 Proteins**

We investigated BAD's relocation between mitochondria and cholesterol-rich domains (rafts). We identified two novel regions in BAD that are responsible for association with lipids (LBD1 and LBD2) and that mediate protein-independent membrane association. Originally, it was proposed that BAD association with mitochondrial membranes takes place via complex formation with the hydrophobic cleft of Bcl-2 or Bcl-X<sub>L</sub>. Lipid binding is required for interaction with Bcl-X<sub>L</sub> in membranes and for full induction of apoptosis. We used biosensor technology to investigate a quantitative manner the binding of mammalian 14-3-3 proteins to phosphorylated BAD and found that they differ in their efficiency to bind BAD and to extract it from membranes.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**—The phospholipids (PC, PE, PS, CL, PA, and PI), SM, cholesterol, benzamidine, leupeptin, aprotinin, phenylmethylsulfonyl fluoride, thiomarin, CHAPS, Nonidet P-40 (Nonidet P-40), and polyclonal anti-actin antibodies were purchased from Sigma. Ceramides (natural sources) were purchased from BION-TREND. Glutathione- Sepharose was from Amersham Biosciences and Ni<sup>2+</sup>-nitrilotriacetic acid-agarose from Qiagen. Cytotoxic necrosing factor (CNF) was obtained (22) and applied as described (23). Phosphospecific antibodies against BAD phosphoseresines 112, 136, and 155 were purchased from Cell Signaling Technology and BIOSOURCE. Rabbit polyclonal BAD antibody (C-20), anti-kinase Aε (C-20), anti-GFP (sc-8334), and 14-3-3 antibodies (K-19 and H-8) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Horseradish peroxidase-conjugated polyclonal antibody (rabbit and mouse IgG) were from Amersham Biosciences. The 25-mer BAD-BH3 peptide used was that described by Petros et al. (24). The phosphorylated version has a phosphoserine at position 155.

**Cloning of BAD, Deletion Mutants, and Site-directed Mutagenesis**—Human BAD full-length and its deletions were PCR-amplified, with primer overhangs containing the restriction sites for cloning. PCR products were cleaved with NdeI and XhoI restriction enzymes and cloned into glutathione S-transferase (GST) fusion vector pGEX-TT-cleaved with the same enzymes. For expression of histidine-tagged BAD in insect cells, the BAD CDNA was cloned into a PFastBacHTA (Invitrogen). Site-directed mutagenesis was done using QuikChange™ (Stratagene) according to the manufacturer’s instructions. Fluorescent versions of BAD used in cell survival assays were prepared by fusing BAD mutants with the C terminus of pEGFP vector (Molecular Probes, Inc.).

**Cell Fractionation**—HEK293 cells transfected with pEGFP-BAD mutants were washed two times with ice-cold PBS. Cell pellets were homogenized in buffer (10 mM Hepes, pH 7.4, and 1 mM EGTA) containing protease inhibitors and centrifuged two times at 700 × g for 10 min at 4 °C to remove nuclei and intact cells. After further centrifugation at 10,000 × g for 25 min at 4 °C, cell pellets containing mitochondria-enriched fraction were washed twice in homogenization buffer and solubilized in Laemmli buffer. To obtain the cytosolic fraction, supernatants were centrifuged at 100,000 × g for 1 h at 4 °C. The light membrane pellet was washed twice in homogenization buffer and solubilized with Laemmli buffer. Protein concentrations of all fractions were determined and equal amounts of total protein have been subjected to SDS-PAGE and immunoblotted.

**Colony Yield Assay**—NIH 3T3 cells were transfected with the indicated plasmids using Lipofectamine. The day after transfection, cells were split, and around 500 cells of each set of transfection were seeded in 6-cm dishes. Colony assays were performed in triplicate by scoring the number of colonies (consisting of at least 20 cells) in the dishes grown for 14 days under selection (450 μg/ml neomycin; Calbiochem). To visualize the growing colonies, cells were washed with PBS, fixed with methanol, and stained with Giemsa dye (Sigma).

**Fluorescence Microscopy**—NIH 3T3 cells transfected with pEGFP-BAD mutants were grown on coverslips for 36 h in 10% fetal calf serum plus Dulbecco’s modified Eagle’s medium. After two washings with ice-cold PBS, cells were fixed for 30 min with 3.7% paraformaldehyde at room temperature. After three washes with PBS, the cells were treated with 0.1 μg/ml Hoechst-33342 dye (Sigma) for 30 min. Excess dye was washed out with PBS (three times) and water. The cells were covered with Mowiol (Calbiochem) and subjected to fluorescence microscopy (Zeiss).

For localization of BAD in raft microdomains, HeLa cells transfected with GFP-BAD were treated with 100 μg/ml CNF for 6 h. Cells were rinsed once with PBS, fixed with 3.7% paraformaldehyde, and rinsed again in PBS. After 10-min quenching with 10 μM NH<sub>4</sub>Cl in PBS, the coverslips were incubated for 2 h with a filipin solution (50 μg/ml in PBS with 10% fetal calf serum). After three washes in PBS, coverslips were mounted with Mowiol and dried. Actin staining was carried out with 0.2 μg/ml rhodamine-phalloidin (Molecular Probes).

PC12 cells were cultured in Dulbecco’s modified Eagle’s medium, 10% horse serum, and 5% fetal calf serum. For the experimental procedure, the cells were cultured for 24 h under low serum conditions and treated with NGF (50 ng/ml) for 2 min. The cells were then fixed with ice-cold methanol and kept at −20 °C until further use. The cells were washed with PBS (25 mM Tris-HCl, pH 7.4, 0.8% NaCl, 0.2% KCl), 10% goat serum, and 0.1% Tween 20. Immunostaining with the indicated antibodies (flotillin-2, cytochrome c oxidase, or mouse anti-BAD) was done for 24 h at 4 °C. Control samples were treated in the absence of the first antibody. Cells were washed three times with PBS containing 10% goat serum and incubated for 30 min with 2 ng/ml Cy-3-coupled goat anti-rabbit antibody (Biomol) or 2 ng/ml Cy-2-coupled anti-mouse antibody (Biomol). Cells were washed again three times with PBS and 10% goat serum and then covered with Mowiol in 50% glycerol/PBS (v/v) and observed under a Leica confocal microscope (TCS; Leica, Heidelberg, Germany).

Immunohistochemical signals were analyzed using Leica Confocal Image software. Single estimations were pooled, and the results were expressed as mean and S.E. Statistical significance of differences was assessed by analysis of variance followed by Bonferroni’s test using Prism (GraphPad, San Diego, CA).

**Mitochondrial Binding Assay**—In order to perform in vitro binding of BAD to mitochondria, these organelles were isolated from HeLa cells according to a standard protocol. Briefly, cells were lysed in a mitochondrial isolation buffer consisting of 220 mM mannitol, 70 mM sucrose, 1 mM EGTA, 2 mM bovine serum albumin, and 20 mM Hepes, pH 7.4, supplemented with a protease inhibitor mixture (Sigma). After homogenization of the cell suspension using a Dounce homogenizer (40 strokes), the postnuclear supernatant was obtained and the mitochondrial pellet was isolated by centrifugation at 12,000 × g for 15 min at 4 °C. Mitochondria were resuspended in a storage buffer containing 250 mM sucrose, 1 mM EGTA, 5 mM sodium succinate, 1 mM ATP, 0.08 mM ADP, 2 mM K<sub>2</sub>HPO<sub>4</sub>, 10 mM Mops, pH 7.2. This protein concentration was determined with a BCA kit (Pierce). Rabbit reticulocyte lysates were produced and radiolabeled by Redivue-Cys-Met<sup>35S</sup>-Mix using the T7 TNT Quick-coupled Transcription-Translation system (Promega) according to the manufacturer’s instructions. For each mitochondrial binding reaction, a mixture of 100 μg of mitochondrial proteins and 5 μl of reticulocyte lysate in storage buffer was used. Mitochondria were incubated at 30 °C for the indicated time and isolated with an additional step of centrifugation. The mitochondrial pellet was rinsed with storage
buffer and resuspended in loading buffer. For quantification, the samples were applied to SDS-PAGE. The radioactivity was detected by an autoradiography enhancer En3hance (PerkinElmer Life Sciences) using ECL films (Amersham Biosciences). The results were quantified using the software ImageJ (National Institutes of Health).

**Tryptic Treatment of Mitochondria**—In order to remove proteins from the surface of the isolated mitochondria, purified preparations were incubated with trypsin. For each treatment, 100 μg of mitochondrial sample were incubated with 20 μg/ml trypsin for 15 min at 4°C. Proteolysis was stopped by the addition of 500 μg/ml soybean trypsin inhibitor. The mitochondria were then isolated and used for binding assays as described above.

**Protein Expression and Purification**—For purification of His$_{6}$-BAD protein, Sf9 cells were infected with baculoviruses at a multiplicity of infection of 5 and incubated for 48 h at 30°C. The Sf9 cell pellets were lysed in 10 ml of Nonidet P-40 lysis buffer containing 50 mM phosphate buffer, pH 8.0, 150 mM NaCl, 10 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 25 mM NaF, 10% glycerol, 0.75% Nonidet P-40, and a mixture of standard protease inhibitors for 45 min with gentle rotation at 4°C. The lysate was centrifuged at 27,000 × g (SS34 rotor, Sorvall centrifuge) for 30 min at 4°C. The supernatants (10 ml) containing His$_{6}$-BAD were incubated with 0.5 ml of Ni$^{2+}$-nitrotriacetic acid-agarose for 2 h at 4°C with rotation. After incubation, the beads were washed three times with Nonidet P-40 buffer (0.2% Nonidet P-40), and His$_{6}$-BAD was eluted with imidazole using a 20–200 mM step gradient.

BAD, Bcl-X$_{I}$, and 14-3-3 proteins were expressed in *Escherichia coli* as GST fusion proteins using pGEX-2T vector (Amersham Biosciences) and purified by glutathione-Sepharose affinity chromatography. 14-3-3 proteins and Bcl-X$_{I}$ were released by thrombin cleavage. GST-Bcl-X$_{I}$ and GST-BAD proteins were purified from 20 ml glutathione using standard protocols. The purity of proteins was validated by SDS-PAGE and staining with Coomassie Blue. For Western blot analysis, the gels were transferred onto nitrocellulose membranes (Schleicher & Schuell) and probed with antibodies as indicated in the figure legends. After washing, the membranes were incubated with specific secondary horseradish peroxidase-conjugated antibodies and detected by ECL (Amersham Biosciences).

**Kinase Activity Assay**—Human GST-BADwt and mutants (20 pmol) were incubated with purified protein kinase A in 50 mM Hepes buffer, pH 7.6, in the presence of 10 mM MgCl$_{2}$, 1 mM dithiothreitol, and 500 μM ATP. The mixture was incubated at 30°C for 30 min, and the reaction was terminated by the addition of Laemmli buffer. The proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. The extent of BAD phosphorylation at serine 155 was detected by a phosphospecific antibody.

**Preparation of Liposomes and Biosensor Measurements**—Large unilamellar vesicles were prepared by the extrusion method using a LiposFast extrusion apparatus (Avestin Inc.) as described (21). To determine quantitatively the interactions between BAD and different 14-3-3 isoforms and for BAD interactions with liposomes, the surface plasmon resonance (SPR) technique was applied. The biosensor measurements were carried out either on Biacore-X or Biacore-J machines (Biacore AB, Uppsala, Sweden) at 25°C. For BAD interactions with 14-3-3 proteins, the biosensor chip CM5 was first loaded with anti-GST antibody using covalent derivatization. Purified GST-14-3-3 proteins were injected in biosensor buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 0.01% Nonidet P-40) at a flow rate of 10 μl/min, resulting in a deposition of ~1200 response units (RU). Next, the purified Sf9 His$_{6}$-BAD was injected at the indicated concentrations. The values for non-specific binding measured in the reference cell were subtracted. To monitor BAD interactions with artificial membranes, liposomes were captured onto the surface of either Pioneer L1 or HPA sensor chips (Biacore). The surface of the sensor chips was first cleaned with 20 mM CHAPS, followed by the injection of liposomes (0.4 mM lipid concentration) at a flow rate of 10 μl/min in 10 mM Hepes, pH 7.4, 150 mM NaCl, and 0.1 mM dithiothreitol, which resulted in a deposition of ~2000–3000 RU for HPA, and 4000–5000 RU for the L1 sensor chip. To prove whether reassociation of analyte occurs in the dissociation phase, reduced concentrations of lipids (40 μM) were also used. To measure the interactions of BAD or BAD mutants with lipids, purified BAD (100–200 mM distilled in biosensor buffer) was applied to the captured liposomes at a flow rate of 10 μl/min. After binding of BAD to lipids, the dissociation was monitored at the same flow rate. At the end of the binding assay, the sensor chip surface was regenerated by injection of 20 mM CHAPS. The evaluation of kinetic parameters was performed by nonlinear fitting of binding data using the BioEvaluation 2.1 software. The apparent association rate ($k_{a}$) and dissociation rate ($k_{d}$) constants were evaluated from the differential binding curves ($F(t) - F(t)$), assuming an $A + B = AB$ association type for the protein-protein interaction. The affinity constant $K_{D}$ was calculated from the equation $K_{D} = k_{d}/k_{a}$.

**RESULTS**

**BAD Associates with Negatively Charged Phospholipids and Cholesterol-rich Liposomes**—Although the primary structure of BAD contains no stretches of hydrophobic amino acids that could function as membrane anchor, we asked whether it could interact with membranes via direct association with lipids. To this end, we explored interactions of purified GST-BAD with artificial lipid membranes resembling plasma and mitochondrial membranes. In addition, we investigated BAD association with liposomes mimicking rafts (i.e., containing high levels of cholesterol and sphingomyelin) (25). Interactions were measured quantitatively by the SPR technique. In contrast to negatively charged lipids (see below), we observed a relatively weak association with liposomes composed of neutral lipids, such as PC, PE, and SM. Therefore, we used liposomes composed of PC/SM/PE (50:13:37 mol %) as a basic lipid mixture in further experiments. When PC is reduced by 20% and substituted by PS, this mixture represents the lipid composition of the inner leaflet of plasma membranes (26). In other vesicles, the PC content was substituted by 10% of PA, PI, CL, ceramides, or cholesterol. As shown in Fig. 1, BAD associated effectively with negatively charged lipids, such as PS, PA, and CL, as well as with cholesterol-rich vesicles. Liposomes consisting of mitochondrial lipids bound BAD with an affinity comparable with PA- and CL-containing vesicles. The mitochondrial membrane represents one of the most strongly negative charged biological membranes (27). Control proteins, such as GST alone or 14-3-3 proteins, displayed only background levels of binding without any specificity for particular lipids (data not shown). For positive control, binding assays were done with raft-like liposomes and purified C-RAF (21). Representative association-dissociation curves are shown in Fig. 1B. The apparent $K_{D}$ values for BAD interactions with various lipid combinations were in the range of 17–69 nM (Table 1). The association rate constants ($k_{a}$) show that the interaction of BAD with cardioliopin, PA, and mitochondrial liposomes is stronger than interactions with PI or ceramide-containing liposomes (Table 1). BAD also bound efficiently to liposomes prepared from soybean lipid extract (asolectin; Fig. 1). To compare the interactions obtained with spheric highly curved liposomal membranes with those with planar lipid monolayers and to guard against possible artifacts caused by asymmetric distribution of lipid components between the inner and outer membrane of liposomes, we
investigated BAD association with lipids immobilized on the surface of the HPA sensor chip. This chip enables formation of stable lipid mono-layers. No significant differences were recorded (Fig. S1).

Two Distinct Domains (LBD1 and LBD2) Differentially Mediate BAD Interactions with Lipids—To localize the putative lipid-interacting domain(s) of BAD, we chose several breakpoints in its primary structure (see Fig. 2A, arrows). The first point was set at the beginning of the BH3-containing helix-4, the second at the end of the BH3 domain, and the third at the beginning of the C-terminal helix-5 (Fig. 2). Proteins truncated at one of the chosen breakpoints (BADΔC36, BADΔC47, BADΔC62, BADΔN102, BADΔN120, and BADΔN131; see Fig. 2B) were expressed as GST fusions in *E. coli* and purified by affinity chromatography. Correct folding of chosen mutants was verified by protein kinase A phosphorylation that is known to phosphorylate preferentially the serine 155 (1). Except for BADΔN120 and BADΔC62 (where Ser-155 was deleted), all other mutants were phosphorylated by protein kinase A at this site (Fig. S2). To investigate whether C-terminal truncations and point mutations of BAD (see below) affect the association with Bcl-X<sub>L</sub>, we performed binding studies using biosensor technology (Fig. S3). In accordance with Petros *et al.* (24), we found that not only the *bona fide* BH3 domain but the entire BH3 helix is required for efficient binding of BAD with Bcl-X<sub>L</sub> (Fig. S3A). BADΔC36 mutants bound
Bcl-XL with nearly the same efficiency as full-length BAD (Fig. S3B).

These interactions were blocked by competition with BAD BH3 peptide (Fig. S3C), verifying the specificity of interactions.

Next, the purified truncated proteins were tested for their ability to associate with lipids as described for wild type BAD (Fig. 1). Two of the C-terminal deletion mutants, BADΔC47 and BADΔC62, completely lost the ability to associate with liposomes (Fig. 3C). On the other hand, the fragment complementary to BADΔC47 (BADΔC62), consisting of 47 C-terminal amino acids downstream of the BH3 domain, associated with lipids effectively (Fig. 3, A and B). BADΔN120 containing both C-terminal α-helices was poorly soluble. Therefore, no experiments toward its lipid association could be done (indicated by N.D. in Fig. 2B).

The lack of lipid binding of BADΔC47 was unexpected, since this BAD fragment contains the complete BH3 domain that is known to possess a pronounced amphipathic character (24). The dissociation rate constants ($k_d$) for binding of the complementary fragment, BADΔN120, to liposomes were lower than those of BADwt, resulting in $K_d$ values between 0.5 and 1.5 nM (Fig. 3B). Thus, BADΔN120 (bearing the last 47 amino acids of BAD) associates with some membranes more tightly than the full-length BAD, which might reflect a regulatory role of the N-terminal part of the BAD protein.

In contrast to breakpoint 2 mutants (BADΔC47 and BADΔN120), both breakpoint 3 mutants (BADΔC36 and BADΔN131) bound lipids effectively (Figs. 2 and 3). However, the lipid binding preferences were strikingly different. BADΔC36 containing the region downstream of BH3 but no helix-5 selectively bound cholesterol-rich liposomes, whereas BADΔN131 showed the opposite pattern (Fig. 3C). The in vitro findings reflect the situation in vivo, because cell fractionation of HEK293 cells expressing C-terminal fragments of BAD (BADΔN131, BADΔN120, and BADΔN102) identified the presence of these proteins in heavy and light membrane fractions (data not shown).

In summary, our analyses revealed that the C-terminal 47 amino acids are sufficient for full lipid association of BAD. Two lipid binding domains were identified within this region that differ in lipid binding.
specifities. The first domain lies between breakpoints 2 and 3 (amino acids 120–131) and will be termed LBD1. The second domain resides in the C-terminal α-helix 5 (amino acids 131–168) and will be termed LBD2. LBD1 mediates selective binding to cholesterol, whereas LBD2 prefers negatively charged lipids.

The Role of Phe-Lys-Lys (FKK) Motif in Lipid Binding—In search for motifs that might be responsible for the lipid binding specificity of LBD1, we focused on Phe-Lys-Lys (FKK) at positions 125–127. This motif is also present in KSR (kinase suppressor of Ras), where it was suggested to be involved in lipid binding (28).
To address the role of individual residues of the FKK motif, we substituted phenylalanine (BAD-AKK) and both lysines (BAD-FAA) by alanines. Determination of lipid binding properties of mutant proteins showed a considerable reduction for both mutants (Fig. 4, A and B). The pattern of binding was remarkably different, however, in that the exchange of phenylalanine (BAD-AKK) affected predominantly binding to cholesterol-containing vesicles, whereas exchange of lysines primarily affected binding to acidic membranes. These findings are consistent with a dual role of this motif in facilitating binding to cholesterol-rich membranes as well as to acidic membranes. Moreover, as previously shown, the presence of this region if not its sequence is required for conformational stability of the BH3 helix and thus association with Bcl-X\textsubscript{L} (24). Our data clearly show that the FKK motif is essential for lipid binding of LBD1. Consistent with this interpretation, a construct missing LBD2 with additional mutation of the FKK motif (BAD\textsubscript{ΔC36-FAA}) completely lost its lipid binding properties (Fig. 4C).

BAD Binds to Protein-depleted Native Membranes—To examine whether BAD binds to native membranes, we used mitochondria isolated from HeLa cells. Full-length BAD and its C-terminally truncated version (BAD\textsubscript{ΔC47}) were synthesized and radiolabeled in the rabbit reticulocyte expression system and co-incubated with mitochondria. In both cases, serine 136 was mutated to alanine in order to prevent 14-3-3-
BAD Association with Liposomes and 14-3-3 Proteins

3-mediated depletion from mitochondria (see Fig. 9). At various time points, the mitochondria were pelleted by centrifugation and analyzed for associated BAD using autoradiography (Fig. 5). Whereas the full-length BAD bound to mitochondria fast and efficiently (t½ ~ 2 min), the BADΔC47-S136A exhibited no measurable binding (Fig. 5). Importantly, BAD binding efficiency did not change upon treatment of mitochondria with trypsin, strongly indicating that BAD binding to mitochondria is not mediated by Bcl-XL or additional protein(s).

Lipid Binding Is Required for Proapoptotic Capacity of BAD—BAD overexpression has been described to neutralize antiapoptotic members of the family, such as Bcl-2 and Bcl-XL. As a consequence, other BH3-only proteins such as Bid and Bim may become displaced and free to activate cytosolic Bax (5). The activation process presumably involves interaction of the BH3 domains with a BH3 binding pocket in Bax that was previously occupied by its C-terminal hydrophobic domain. This process leads to translocation of the cytosolic Bax to the mitochondrial membrane, its oligomerization, and function as a pore (3, 4).

To investigate the biological properties of BAD and its derivatives with impaired lipid binding in vivo, we performed two types of cell survival assays. First, in a colony yield assay, NIH 3T3 cells were stably transfected with the vectors bearing either the wild type BAD or different BAD mutants lacking either C- or N-terminal parts of different size. After 14 days of growth under selection, the colonies were stained and counted (Fig. 6). In contrast to vector-transfected cells, less than one-third of plated cells (150 cells versus 496 cells of 500 cells) formed colonies in the case of full-length BAD. BADΔC36, which still binds lipids via LBD1, yielded comparable numbers of colonies. Similarly, assay of BADΔN102 (which contains BH3 in combination with both LBDs) showed a degree of reduction indistinguishable from full-length BAD. On the other hand, BADΔC47 and BADΔC62 mutants formed significantly more colonies, indicating that the proapoptotic function of these mutants is impaired. Mutants possessing only the C-terminal LBDs but missing BH3 showed growth comparable with vector control. Mutation of the FKK motif reduced the proapoptotic activity of both full-length BAD and BADΔC36, consistent with its effect on lipid binding. For the second assay, we determined the yield of GFP-positive cells after transient transfection of NIH 3T3 with consistent results (data not shown). We conclude that both elements (i.e. BH3 domain and lipid binding domains) are essential for proapoptotic activity of BAD.

BAD Interaction with Bcl-XL in Membranes Suggests a Potential Receptor Function—Jeong et al. (6) showed that Bcl-XL forms cytosolic homodimers by cross-linking of the hydrophobic C termini with the BH3 binding grooves. The data also suggested a role for the BH3 domain of BAD in membrane translocation and thus activation of Bcl-XL through displacement of C termini from the BH3 binding grooves. This mechanism is analogous to BAX activation by “activator” class BH3-only proteins Bid and Bim (3).

In order to examine the ability of wild type and mutant BAD to mediate membrane translocation of Bcl-XL in our liposome system, we first measured Bcl-XL/BAD interactions in solution as described (Fig. S3). Lipid binding of BAD was dispensable for complex formation with Bcl-XL in solution. Whereas the exact sequence of the FKK motif, which is essential for lipid binding, was not required for association with Bcl-XL in solution, the presence of three amino acids in this position is necessary to stabilize the BH3 helix. Very different results were obtained when Bcl-XL/BAD interaction was measured in the presence of liposomes. In the absence of BAD, Bcl-XL was a poor lipid binder, whereas Bcl-XLΔΔC21 did not bind at all (Fig. 7A). The addition of BAD BH3 peptide slightly increased binding of Bcl-XL. The binding of Bcl-XL to membranes was greatly accelerated by prebound BADwt (Fig. 7B) and completely eliminated by the addition of BAD BH3 peptide to this mixture. The ability of BAD to facilitate Bcl-XL membrane translocation required LBD2 of BAD (compare BADwt and BADΔC36 in Fig. 7C) and the C-terminal transmembrane helix of Bcl-XL (Fig. 7B), although both proteins interacted readily in solution (Fig. 7E). We showed (see Fig. 4) by substitutions of phenylalanine and lysines in the FKK motif of LBD1 that phenylalanine plays a central role in recognition of cholesterol-rich lipid domains (rafts). How does this phenomenon affect the recruitment of Bcl-XL to membranes? As demonstrated in Fig. 7D, only in the presence of rafts is the association of Bcl-XL to BAD inhibited significantly. These data underscore the dual role of LBD1 in both lipid binding specificity and effective recruitment of Bcl-XL. This lack of binding may provide a mechanism to prevent sequestration of Bcl-XL to inappropriate cellular membranes (e.g. cholesterol-rich regions).
We conclude that for specific binding of BAD to Bcl-X\(_L\) in a given membrane environment, both an intact BH3 domain and functional C-terminal lipid binding domain are required. The differential effects of the BAD\(\Delta C36\) in solution binding versus binding in the presence of liposomes may result from occupation of the FKK motif by lipids. The cholesterol-bound FKK region would presumably not be able to bind Bcl-X\(_L\).

**BAD Interaction with Membranes Is Independent of Its Phosphorylation**—BAD protein function and localization are regulated by phosphorylation. Therefore, we asked whether phosphorylation of BAD has an effect on its binding to lipids. Histidine-tagged BAD (His\(_6\)-BAD) was expressed and purified in \(Sf\) insect cells. Whereas BAD protein purified from \(E. coli\) was not phosphorylated,\(^4\) His\(_6\)-BAD from \(Sf\) cells was strongly phosphorylated at serines 75, 99, and 118 (corresponding to serines 122, 136, and 155 of mouse BAD, respectively; Fig. S4). We determined the level of phosphorylation at serine 136 (\(\geq68\)% and serine 155 (\(\geq72\)% by quantifying the binding of \(Sf\) His\(_6\)-BAD to 14-3-3 proteins (Fig. 10) and Bcl-X\(_L\) (Fig. S3). The lipid binding studies with \(Sf\) His\(_6\)-BAD (Fig. 8) resulted in binding profiles that were very similar to unphosphorylated GST-BAD from \(E. coli\), demonstrating that BAD phosphorylation does not influence its ability to interact with membranes. The same results were obtained using a lipid binding assay followed by gel filtration through Sepharose CL-4B columns and detection with phosphospecific antibodies (see Ref. 21).

**Complex Formation with 14-3-3 Prevents BAD Association with Mitochondrial Membranes and Promotes Its Association with Cholesterol-rich Liposomes**—It has been proposed that in vivo phosphorylation of BAD disrupts the BAD-Bcl-X\(_L\) complex and enables formation of a BAD-14-3-3 complex with the consequence of cytosolic sequestration of BAD (8). Since we have shown here that BAD association with lipids does not require other interactors and is phosphorylation-independent (see Figs. 1, 3, and 8), we asked further whether BAD phosphorylation might function as a signal for its removal from the membranes by 14-3-3 proteins. To test this assumption, we employed His\(_6\)-BAD from \(Sf\) cells that is highly phosphorylated at the 14-3-3 binding site Ser-136 (Fig. S4) and binds 14-3-3 proteins effectively in solution. In the experiment shown in Fig. 9, phosphorylated BAD was first bound to the immobilized liposomes. Next, purified 14-3-3 proteins were injected as indicated. Surprisingly, out of the seven isoforms, only the 14-3-3\(\gamma\), \(-\eta\), and \(-\tau\) were able to deplete BAD efficiently from the mitochondrial liposomes (Fig. 9A, black bars). 14-3-3\(\alpha, -\beta,\) and \(-\gamma\) were less efficient, whereas 14-3-3\(\xi\) was inefficient. In contrast, 14-3-3 proteins failed to deplete BAD from cholesterol-containing liposomes (Fig. 9A, gray bars). To exclude artifacts that may be caused by ingredients in the 14-3-3 protein samples, a control flow cell containing only lipid vesicles was run simultaneously. As shown in Fig. 9B, in this channel only background binding (\(\approx50\) RU) of 14-3-3\(\gamma\) was recorded, and the lipid base line remained constant. These experiments clearly demonstrate that phosphorylated BAD can be specifically released from mitochondrial but not from cholesterol-rich membranes by a subset of 14-3-3 proteins. Importantly, using nonphosphorylated BAD, no 14-3-3-mediated release from any liposomes was observed (Fig. 9A, right).

Next, we investigated whether preformed BAD-14-3-3 complexes associate with membranes with the same affinity as free BAD. Phosphorylated His\(_6\)-BAD was preincubated with 14-3-3 proteins and injected

---

\(^4\) U. E. E. Rennefahrt, M. Hekman, S. Albert, A. Fischer, J. Fueller, and U. R. Rapp, submitted for publication.

---

**FIGURE 6. Colony formation assay of cells expressing BAD mutants.** NIH 3T3 cells were transfected with pcDNA3 bearing human BAD or indicated mutants and about 500 cells were seeded in Petri dishes. After 2 weeks growth under selection, the formed colonies were stained (A) and counted (B). The experiment has been repeated three times. It is apparent that both BH3 domain and lipid binding are required for pro-apoptotic function of BAD.
FIGURE 7. Formation of BAD-Bcl-X<sub>L</sub> complex in the lipid environment. A, wild type (wt) Bcl-X<sub>L</sub> associates with liposomes less efficiently than BAD<sub>wt</sub>. The L1 sensor chip was loaded with liposomes mimicking mitochondrial lipids (~5000 RU). Next, purified full-length BAD (200 nM) or Bcl-X<sub>L</sub> (200 nM) was injected in the absence or presence of 10 μM BAD BH3 peptide. The C-terminally truncated mutant Bcl-X<sub>L</sub> ΔC21 (200 nM) did not exhibit significant binding to lipids. B, interaction between BAD and Bcl-X<sub>L</sub> in a lipid environment requires intact Bcl-X<sub>L</sub>. The L1 sensor chip was loaded with liposomes as described in A. Next, purified BAD<sub>wt</sub> (200 nM) was immobilized on liposomes. Finally, purified Bcl-X<sub>L</sub> ΔC21 (100 nM)
to the immobilized lipid vesicles. Consistent with the membrane depletion data (Fig. 9A), the BAD-14-3-3 complex associated effectively only with cholesterol-containing liposomes (Fig. 9C, right bars). In contrast, the presence of negatively charged lipids, such as CL, PA, PS, and the mitochondrial lipids, in liposomes reduced binding of BAD-14-3-3 (Fig. 9C). These results strongly suggest that the BAD-14-3-3 complex exhibits higher affinity for cholesterol-rich and diminished affinities for mitochondrial or other negatively charged membranes. To investigate whether 14-3-3 proteins remain bound to or dissociate from BAD upon membrane association, we incubated the BAD-14-3-3 complex with different liposomes and analyzed the lipid fractions for associated proteins after gel filtration (21). This experiment revealed that, similar to RAF, the BAD-14-3-3 complex dissociates nearly completely upon association with liposomes (data not shown).

Isoform Specificity of 14-3-3 Binding to Phosphorylated BAD—The observed differences in efficiencies of BAD depletion from liposomes prompted us to investigate binding of 14-3-3 isoforms to phosphorylated BAD in the absence of lipids. As shown in Fig. 10A, significant differences in the binding of 14-3-3 isoforms to $S^9$ His$_6$-BAD have been recorded. Strikingly, 14-3-3 isoforms that are efficient in BAD depletion from liposomes ($\zeta$, $\eta$, and $\tau$, see Fig. 9A) exhibited significantly lower dissociation rates (Fig. 10A). To quantify these differences, we followed the kinetics of BAD association with 14-3-3$\zeta$ and 14-3-3$\gamma$, two isoforms that exhibit opposite behavior with respect to BAD removal from membranes. GST-14-3-3 proteins were immobilized on the CM5 chip coated with anti-GST antibodies, and the association-dissociation curves with purified His$_6$-BAD in the concentration range of 40–320 nM were monitored (Fig. 10, B and C). The apparent association ($k_a$) and dissociation rate constants ($k_d$) differed significantly (Fig. 10, compare B and C). Consequently, the calculated $K_D$ values reveal that affinity of 14-3-3$\zeta$ is >11-fold stronger than that of 14-3-3$\gamma$ ($K_D = 1.2$ versus 13.9 nM, respectively). These results may explain the observed high efficiency of 14-3-3$\zeta$ in the depletion of BAD from the membranes (Fig. 9A).
differences of more than 1 order of magnitude in $K_D$ values might be due to the fact that 14-3-3ζ, -η, and -τ but not other isoforms make molecular contacts to additional (unphosphorylated) residues of BAD. Indeed, it has been shown that 14-3-3ζ forms a complex with serotonin N-acetyltransferase, interacting not only with the phosphopeptide binding groove but also with other parts of the molecule (30).
BAD Relocates from Mitochondria to Cholesterol-rich Structures (Rafts) in Response to Stimulation with Growth Factor—To demonstrate relocation of BAD to cholesterol-rich membrane domains in vivo, we observed the distribution of endogenous BAD in serum-starved and NGF-stimulated PC12 cells. Data shown in Fig. 11 and Fig. S5 clearly demonstrate that the initial co-localization of BAD with mitochondria (starvation conditions) disappears after NGF treatment (Fig. 11A, top panels). Concomitantly, a massive association with cholesterol-rich structures occurs (Fig. 11A, bottom panels). Cholesterol-rich structures were detected by an established raft marker flotillin-2 (31). Statistical analysis of colocalization data (Fig. 11B) confirmed that the observed changes in BAD distribution are highly significant (p < 0.001). These data are consistent with our model of BAD function published recently (32) (see “Discussion”).

Lamellipodia have been reported to be rich in cholesterol that can be decorated by filipin (23). To investigate whether BAD colocalized with these structures, HeLa cells were transiently transfected with GFP-BAD. After treatment with CNF, a potent activator of Rac-PAK pathway (22, 23), the cells were examined by fluorescence microscopy. Under these conditions, a significant colocalization between filipin and GFP-BAD was observed (Fig. 12, A and B). This colocalization occurred indeed in areas of membrane ruffling, since GFP-BAD also colocalizes with actin at these sites. Efficient induction of lamellipodia is evident from actin staining (Fig. 12D). Taken together, our binding experiments and cell imaging data strongly suggest that BAD binds to cholesterol-rich membrane domains under prosurvival conditions.

DISCUSSION

The current dogma holds that BAD association with mitochondrial membranes takes place via complex formation with the hydrophobic groove of Bcl-2 or Bcl-X<sub>L</sub> (2). We show here that membrane localization of BAD does not require additional protein components and in fact mediates membrane translocation of Bcl-X<sub>L</sub>. The C-terminal part of BAD is sufficient for membrane binding. Two segments with different lipid binding preferences were identified that are responsible for this interaction: (i) LBD1 located in the proximity of the BH3 domain (amino acids 122–131) and (ii) LBD2, the putative C-terminal α-helix-5 (see also Fig. 2). Phosphorylation-regulated 14-3-3 protein binding (Fig. 10A) may expose the cholesterol-preferring LBD1 and bury the LBD2, thereby mediating translocation of BAD to raft-like microdomains.

Previously, a synthetic peptide (25-mer) consisting of the BAD BH3 domain plus flanking amino acids was assayed for interactions with the hydrophobic groove of Bcl-X<sub>L</sub> using NMR spectroscopy (24). The authors showed that this peptide forms an amphipathic α-helix, interacting with Bcl-X<sub>L</sub> via its hydrophobic face. A 16-mer peptide representing the bona fide BH3 domain of BAD bound Bcl-X<sub>L</sub> only poorly. The K<sub>D</sub> value of the 16-mer was higher by 2 orders of magnitude than that of the 25-mer. The regions proximal to BH3 were shown to stabilize the helical conformation of BH3, whereas mutations of individual residues in the flanking regions had almost no effect on Bcl-X<sub>L</sub> binding. Here we demonstrate that the C-terminal part of the BH3 amphipathic α-helix, but not its N-terminal part, displays pronounced lipid binding with a preference for cholesterol-rich liposomes (see Figs. 1–3). This domain is designated...
LBD1. The FKK motif that is part of the BH3 amphipathic α-helix plays a central role in lipid binding of LBD1. Mutations of phenylalanine or lysines of this motif diminished the lipid association of full-length BAD and abolished that of BAD/H9004C36 (lacking LBD2) completely (Fig. 4). In contrast to LBD1, LBD2 exhibits high affinity for negatively charged lipids. These findings are consistent with a dual role of the FKK motif in LBD1 in regulating binding to cholesterol-rich membranes and in stabilizing complex formation with Bcl-XL in the membrane.

What are the consequences of direct BAD binding to membranes? Our experiments with Bcl-XL suggest that BAD might have a receptor function important for the membrane translocation of Bcl-XL. Earlier experiments by Jeong et al. (6) suggested the following sequence of steps for BAD-induced membrane translocation of Bcl-XL: (i) BAD-BH3 domain triggers the displacement of the C-tail of Bcl-XL from its BH3 pocket, and (ii) the exposed C-tail now inserts into the mitochondrial membrane, pulling BAD along. Our data clearly show that a form of BAD that still binds Bcl-XL in solution but with compromised C-terminal lipid binding (BADΔC36 or BADΔC36-FAA) no longer drives membrane translocation of Bcl-XL. This finding is in agreement with the data by Jeong et al. (6) on the ability of BAD to mediate membrane translocation of Bcl-XL. It is novel in that it shows that membrane-prebound BAD is able to target Bcl-XL to mitochondria. The C-terminal lipid binding domains of BAD are also required for this process.

How does BAD promote apoptosis? We show here that under survival conditions, 14-3-3-bound BAD is accumulated in raft microdomains, where 14-3-3 is stripped off the protein. When the cells are subsequently starved, dephosphorylation of BAD in rafts can take place, and this form of BAD, which has a very high affinity for acidic membranes, would accumulate at mitochondria (see below) (32). At this site, BAD might displace activator class BH3-only proteins (Bid and Bim) from resident prosurvival Bcl members (Bcl-XL, Bcl-2, and Bcl-w). The BH3 domain of free activators would then displace the C-tail of Bax from its BH3-binding pocket and thereby trigger oligomerization and pore formation as predicted by the model of Green and Kroemer (33). The latter model is largely based on the data of Chen et al. (4) and Kuwana et al. (3), who classified BH3-only proteins according to their range of prosurvival protein targeting (4) and ability to activate Bax (3). Briefly, these data demonstrate that BAD belongs to the group of BH3-only proteins with a restricted set of prosurvival targets, whereas Bid, Bim, and Puma interact with all five targets tested. It is probably not possible to predict which BH3-only proteins might be displaced by membrane-bound BAD from heterodimers with prosurvival proteins Bcl-2, Bcl-XL, or Bcl-w on the basis of published data (4). The differential targeting experiments with prosurvival Bcl-2 proteins and their BH3-only ligands were done with C-terminally truncated proteins and in solution, conditions that we have shown to be potentially misleading. A case in point is our finding that Bcl-XLΔC21, although readily binding BAD in solution, does not show interaction with membrane-bound BAD (Fig. 7, compare B and E). Functional evaluation of BAD-Bcl-XL complexes, therefore, awaits future experiments with intact BH3-only proteins at the membrane.

Considering the differences in lipid binding specificities of the two LBDs and differences between free or 14-3-3-bound BAD, it is tempting...
to speculate that interplay of the two lipid binding domains could add to the regulation of BAD. Possibly, free BAD protein folds in a conformation where the LBD2 is exposed, and the whole protein possesses high affinity for negatively charged mitochondrial membranes. Conformational changes caused by association with 14-3-3 as a consequence of Ser-136/Ser-112 phosphorylation (see below) may expose the cholesterol-prefering LBD1 and bury C-terminal helix 5. The side chain of the phenylalanine present in the FKK motif could play a role in the interaction with cholesterol-rich membranes via hydrophobic stacking with the planar assembly of cholesterol molecules. In line with this, mutation of the phenylalanine residue in FKK motif (to alanine) reduced the binding efficiency to cholesterol-rich membranes (Fig. 4). Regulated exposure of LBD1 would explain why 14-3-3-bound but not free BAD binds to cholesterol-rich membranes with high affinity. The observed lipid binding properties are apparently important for the proapoptotic effects of BAD, since mutants lacking both lipid binding domains exhibited significantly lower proapoptotic potency compared with wild type BAD as demonstrated in our in vivo assay (Fig. 6). In line with this finding, the expression of BADΔN102 reduced cell survival similar to BAD wild type (Fig. 6). This indicates that the fragment consisting of BH3 in combination with LBD1 and LBD2 is sufficient for full proapoptotic activity of BAD. In principle, these findings are consistent with the model proposed by Datta et al. (8), in which binding of 14-3-3 induces a conformational change, leading to release of Bcl-XL and subsequent phosphorylation of Ser-155. Our data on lipid specificity of BAD indicate that this 14-3-3-mediated conformational change may play an important role in membrane recognition, which adds a new dimension to this model. Furthermore, we demonstrate that membrane-bound and phosphorylated BAD can be effectively extracted by a subset of 14-3-3 proteins, except from cholesterol-rich membranes. Consistently, preformed BAD-14-3-3 complex binds preferentially to cholesterol-rich structures resembling rafts but poorly to mitochondrial membranes. Importantly, 14-3-3 is released during this process, so that 14-3-3-free BAD may become susceptible to dephosphorylation and subsequent change of its binding preferences (to lipids and/or proteins). Our data on raft association of BAD in vivo (Figs. 11 and 12) strongly support this model and are in accordance with the observations from Rebollo’s laboratory (29, 34), who reported that BAD co-localizes with lipid rafts in IL-4-stimulated T-cells and associates with mitochondria in IL-4-deprived cells.

In extension to previous observations (35), we found, testing all mammalian 14-3-3 isoforms, that these proteins associate with BAD in a pronounced isoform-specific manner. These differences are reflected in the pattern of efficiency with which they remove BAD from mitochondrial membranes (see Figs. 9 and 10 for comparison). Based on our results and published data on BAD phosphorylation (14), its association with 14-3-3 proteins (7, 8, 36), and localization of BAD with lipid rafts (29, 34, 37), we propose an updated model for BAD function (32). Growth factor stimulation enhances BAD phosphorylation at serines 112, 136, and 155. Isoform-specific binding of 14-3-3 proteins to phosphorylated BAD results in temporary sequestration of the BAD-14-3-3 complex in the cytosol. BAD-14-3-3 complex associates preferentially with cholesterol-rich membranes (Fig. 9C), consistent with the in vivo observations (Figs. 11 and 12). Dissociation of 14-3-3 proteins from BAD upon binding to cholesterol-rich membranes presumably allows dephosphorylation at phosphoserines 112, 136, and 155. At this stage, BAD may diffuse from rafts within the plane of the membrane to other microdomains (such as liquid-disordered phase) where they may accumulate. Under apoptotic conditions, BAD might relocate to mitochondria and neutralize Bcl-XL function.

Thus, under steady-state conditions three populations of BAD in the cell may exist: one associated with mitochondria (probably as a complex with other members of the Bcl-2 family of proteins), a second associated with rafts or other lipid domains, and a third that forms a transition state in the cytosol where BAD is complexed with 14-3-3 proteins. Taken together, the identification of C-terminal lipid binding domains in BAD alters our view on the function of this sentinel of cell death regulation and highlights the role of lipid interactions in the regulation of Bcl-2 family proteins.

Acknowledgments—We thank R. Metz and B. Bauer for excellent technical assistance. We are indebted to A. Aitken and K. Rittinger for the generous gift of 14-3-3 expression vectors.

REFERENCES
1. Gross, A., McDonnell, J. M., and Korsmeyer, S. J. (1999) Genes Dev. 13, 1899–1911
2. Yang, E., Zha, J., Jockel, J., Boise, L. H., Thompson, C. B., and Korsmeyer, S. J. (1995) Cell 80, 285–291
3. Kuwana, T., Boucher-Hayes, L., Chipuk, J. E., Bonzon, C., Sullivan, B. A., Green, D. R., and Newmeyer, D. D. (2005) Mol. Cell 17, 525–535
4. Chen, L., Willis, S. N., Wei, A., Smith, B. J., Fletcher, J. I., Hinds, M. G., Colman, P. M., Day, C. L., Adams, J. M., and Huang, D. C. (2005) Mol. Cell 17, 393–403
5. Green, D. R. (2005) Cell 121, 671–674
6. Jeong, S. Y., Gaume, B., Lee, Y. J., Hsu, Y. T., Ryu, S. W., Yoon, S. H., and Youle, R. J. (2004) EMBO J. 23, 2146–2155
7. Zha, J., Harada, H., Yang, E., Jockel, J., and Korsmeyer, S. J. (1996) Cell 87, 619–628
8. Datta, S. R., Katsov, A., Hu, L., Petros, A., Fesik, S. W., Yaffe, M. B., and Greenberg,
BAD Association with Liposomes and 14-3-3 Proteins

M. E. (2000) Mol. Cell 6, 41–51
9. Rapp, U. R., Rennefahrt, U., and Troppmair, J. (2004) Biochim. Biophys. Acta 1644, 149–158
10. Wang, H. G., Takayama, S., Rapp, U. R., and Reed, J. C. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7063–7068
11. Jin, S., Zhuo, Y., Guo, W., and Field, J. (2005) J. Biol. Chem. 280, 24698–24705
12. Wang, H. G., Rapp, U. R., and Reed, J. C. (1996) Cell 87, 629–638
13. Harada, H., Becknell, B., Wilm, M., Mann, M., Huang, L. J., Taylor, S. S., Scott, J. D., and Korsmeyer, S. J. (1999) Mol. Cell 3, 413–422
14. Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M. E. (1997) Cell 91, 231–241
15. Schurmann, A., Mooney, A. F., Sanders, L. C., Sells, M. A., Wang, H. G., Reed, J. C., and Bokoch, G. M. (2000) Mol. Cell Biol. 20, 453–461
16. Gotz, R., Wiese, S., Takayama, S., Camarero, G. C., Rossoll, W., Schweizer, U., Troppmair, J., Jablonka, S., Holtmann, B., Reed, J. C., Rapp, U. R., and Sendtner, M. (2005) Nat. Neurosci. 8, 1169–1178
17. Martinez-Senac Mdel, M., Corbalan-Garcia, S., and Gomez-Fernandez, J. C. (2002) Biophys. J. 82, 235–243
18. del Mar Martinez-Senac, M., Corbalan-Garcia, S., and Gomez-Fernandez, J. C. (2001) Biochemistry 40, 9983–9992
19. del Mar Martinez-Senac, M., Corbalan-Garcia, S., and Gomez-Fernandez, J. C. (2000) Biochemistry 39, 7744–7752
20. Kuwana, T., Mackey, M. R., Perkins, G., Ellisman, M. H., Latterich, M., Schneider, R., Green, D. R., and Newmeyer, D. D. (2002) Cell 111, 331–342
21. Helman, M., Hamm, H., Villar, A. V., Bader, B., Kuhlmann, J., Nickel, J., and Rapp, U. R. (2003) J. Biol. Chem. 277, 24090–24102
22. Aktories, K., and Barbieri, J. T. (2005) Nat. Rev. Microbiol. 3, 397–410
23. Kerkhoffs, E., Leberfinger, C. B., Schmidt, G., Aktories, K., and Rapp, U. R. (2002) Biochim. Biophys. Acta 1589, 151–159
24. Petros, A. M., Netteheiem, D. G., Wang, Y., Olepniczak, E. T., Meadows, R. P., Mack, J., Swift, K., Matayoshi, E. D., Zhang, H., Thompson, C. B., and Fesik, S. W. (2000) Protein Sci. 9, 2528–2534
25. Simons, K., and Toomre, D. (2000) Nat. Rev. Mol. Cell. Biol. 1, 31–39
26. Gennis, R. B. (1988) Biomembranes: Molecular Structure and Function, Springer, New York
27. Hovius, R., Thijsen, J., van der Linden, P., Nicolay, K., and de Kruijff, B. (1993) FEBS Lett. 330, 71–76
28. Andresen, B. T., Rizzi, M. A., Shome, K., and Romero, G. (2002) FEBS Lett. 531, 65–68
29. Aylon, Y., Fleischer, A., Cayla, X., Garcia, A., and Rebollo, A. (2002) J. Immunol. 168, 3387–3393
30. Obsil, T., Ghirlando, R., Klein, D. C., Gangoily, S., and Dyda, F. (2001) Cell 105, 257–267
31. Stuermer, C. A., Lang, D. M., Kirsch, F., Wiechers, M., Deininger, S. O., and Plattner, H. (2001) Mol. Biol. Cell 12, 3031–3045
32. Drexler, H. C. A., Galmine, A., Helman, M., Albert, S., and Rapp, U. R. (2006) in Apoptosis and Cancer Therapy (Debatin, K.-M. and Fulda, S., eds) pp. 490–513, Wiley-VCH, Weinheim, Germany
33. Green, D. R., and Kroemer, G. (2005) J. Clin. Invest. 115, 2610–2617
34. Fleischer, A., Ghadiri, A., Dessauge, F., Duhamel, M., Cayla, X., Garcia, A., and Rebollo, A. (2004) Mol. Cancer Res. 2, 674–684
35. Subramanian, R. R., Masters, S. C., Zhang, H., and Fu, H. (2001) Exp. Cell Res. 271, 142–151
36. Masters, S. C., Yang, H., Datta, S. R., Greenberg, M. E., and Fu, H. (2001) Mol. Pharmacol. 60, 1325–1331
37. Garcia, A., Cayla, X., Fleischer, A., Guergnon, J., Alvarez-Franco Canas, F., Rebollo, M. P., Roncal, F., and Rebollo, A. (2003) Biochimie (Paris) 85, 727–731