

## FLIP Protects against Hypoxia/Reoxygenation-Induced Endothelial Cell Apoptosis by Inhibiting Bax Activation

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**Hypoxia/reoxygenation causes cell death, yet the underlying regulatory mechanisms remain partially understood. Recent studies demonstrate that hypoxia/reoxygenation can activate death receptor and mitochondria-dependent apoptotic pathways, involving Bid and Bax mitochondrial translocation and cytochrome *c* release. Using mouse lung endothelial cells (MLEC), we examined the role of FLIP, an inhibitor of caspase 8, in hypoxia/reoxygenation-induced cell death. FLIP protected MLEC against hypoxia/reoxygenation by blocking both caspase 8/Bid and Bax/mitochondrial apoptotic pathways. FLIP inhibited Bax activation in wild-type and Bid<sup>-/-</sup> MLEC, indicating independence from the caspase 8/Bid pathway. FLIP also inhibited the expression and activation of protein kinase C (PKC) ( $\alpha$ ,  $\zeta$ ) during hypoxia/reoxygenation and promoted an association of inactive forms of PKC with Bax. Surprisingly, FLIP expression also inhibited death-inducing signal complex (DISC) formation in the plasma membrane and promoted the accumulation of the DISC in the Golgi apparatus. FLIP expression also upregulated Bcl-X<sub>L</sub>, an antiapoptotic protein. In conclusion, FLIP decreased DISC formation in the plasma membrane by blocking its translocation from the Golgi apparatus and inhibited Bax activation through a novel PKC-dependent mechanism. The inhibitory effects of FLIP on Bax activation and plasma membrane DISC formation may play significant roles in protecting endothelial cells from the lethal effects of hypoxia/reoxygenation.**

Ischemia caused by arterial occlusion, shock, transplantation, or respiratory failure often causes increased cell death. Ischemic lungs suffer profound losses of ATP due to the reduced availability of oxygen and nutrients and concomitant inhibition of oxidative phosphorylation and anaerobic glycolysis (27). Latent, but potentially lethal, ischemic damage may cause cells in different regions of the lung to sustain reperfusion injury when reoxygenated by restitution of blood flow. The restoration of oxygen and nutrients during reperfusion regenerates the ATP supply, which may result in the initiation of apoptosis, an energy-dependent form of cell death, in cells damaged during ischemia (5). Cell death may also arise as a secondary consequence of inflammation around dead tissue. The inflammatory response can play a deleterious role in ischemia/reperfusion (I/R)-induced lung injury (32). In cell culture systems, the hypoxia/reoxygenation (H/R) model is often used to study the effects of I/R *in vitro* (27).

Two main apoptotic pathways have been defined in endothelial cells, an extrinsic pathway mediated by death receptor family proteins (i.e., Fas) and an intrinsic pathway mediated by the mitochondria. We have previously demonstrated that the initiation of the apoptotic process of cell death by H/R involves both pathways (40, 41). The expression of Fas ligand (FasL) during H/R may trigger Fas-dependent death pathways (37, 14), characterized by the formation of the death-inducing signaling complex (DISC). The recruitment of caspase 8 to the

DISC followed by its activation leads to the subsequent activation of effector caspases. The extrinsic pathway can be amplified by the caspase 8-dependent activation of Bid, leading to mitochondrial damage and release of apoptogenic factors. Since mammalian cells depend on mitochondria for long term viability, H/R may cause cell death through irreparable mitochondrial damage (27). In human fetal alveolar type II epithelial cells, the antiapoptotic proto-oncogene Bcl-2 displayed maximum abundance in hypoxia and mild reoxygenation. With increasing partial O<sub>2</sub> pressure, the Bcl-2 expression declined with reciprocal increase in Bax, a proapoptotic Bcl-2 family member (9). Hypoxia also induced a time-dependent mitochondrial translocation of Bax, with the subsequent release of cytochrome *c* and apoptotic cell death upon reoxygenation (28). Bcl-2 and Bax reciprocally control apoptosis by inhibiting or stimulating, respectively, mitochondrial cytochrome *c* release. Cytosolic cytochrome *c* and Apaf-1 cooperatively activate initiator caspase 9, which triggers a caspase cascade leading to apoptosis (2).

FLIP, also known as Fas-associated death domain (FADD) interleukin-1 $\beta$ -converting enzyme (FLICE)-like inhibitory protein has been characterized as an inhibitor of apoptosis induced by death receptors such as Fas or the tumor necrosis factor-related apoptosis-inducing ligand receptors. A human cellular homolog of v-FLIP was found and termed cellular FLICE-inhibitory protein (c-FLIP; also called FLAME-1, I-FLICE, Casper, CASH, MRIT, CLARP, and usurpin) (20, 25). The c-FLIP gene localizes to chromosome 2q33-34 in a cluster of 200 kb that includes caspase 8 and caspase 10, suggesting that these genes evolved by duplication (24). Multiple splice variants of c-FLIP have been found, but so far only two, des-

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ignated c-FLIP<sub>S</sub> and c-FLIP<sub>L</sub>, could be detected at the protein level (20, 24). c-FLIP<sub>L</sub> contains tandem death effector domains and a caspase-like domain which lacks amino acid residues that are critical for caspase activity. c-FLIP<sub>S</sub> resembles its viral counterparts, consisting of two death effector domains and a truncated C terminus that differs from c-FLIP<sub>L</sub> (1). The mechanisms of cell death attenuation by c-FLIP are not completely understood. c-FLIP, acting as a potential competitive inhibitor, precludes the recruitment of caspase 8 to the DISC and thereby prevents its activation (1, 18, 20, 24–25). This hypothesis is supported by the fact that overexpression of the viral homolog v-FLIP-E8 led to its association with the DISC and the inhibition of caspase 8 recruitment and activation (1).

Previously, we have shown that overexpression of exogenous human FLIP<sub>L</sub> significantly inhibited cell death after H/R stress in mouse lung endothelial cells (MLEC) (41). In the same model, cytoprotection against H/R stress conferred by hepatocyte growth factor was associated, in part, with the stimulation of endogenous FLIP expression (41). Since the mechanisms by which FLIP protects against H/R-induced endothelial cell apoptosis are not fully understood, the present study aimed to define these in greater detail. Here, we found that, in addition to inhibiting the recruitment of caspase 8 to the DISC, FLIP<sub>L</sub> can provide protection against both extrinsic and intrinsic apoptotic pathways. FLIP<sub>L</sub> protected against extrinsic (Fas/caspase 8-dependent) apoptosis by blocking DISC translocation from the Golgi complex to the plasma membrane and also protected against intrinsic (Bax/mitochondria-dependent) apoptosis by inhibiting Bax activation through a protein kinase C (PKC)-dependent pathway.

#### MATERIALS AND METHODS

**Chemicals and reagents.** Antibodies anti-Bax, anti-Bid, anti-caspase 8, anti-caspase 3 (H277), anti-cytochrome *c* (A8), anti-Bcl-X<sub>L/S</sub>, anti-Fas, anti-PKC (A3), anti-PKC $\alpha$  (H7), and anti-PKC $\zeta$  (H1) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-Bax 6A7 antibody was from BD PharMingen (San Diego, CA). Adenovirus-human FLIP<sub>L</sub> (Hu-FLIP<sub>L</sub>), adenovirus-*lacZ*, and adenovirus-*crmA* were supplied by the Center for Biotechnology and Bioengineering, University of Pittsburgh (www.vectorcore.pitt.edu). The protein kinase C inhibitor GF109203X, digitonin, and all other chemicals were from Sigma (St. Louis, MO).

**Isolation and culture of MLEC.** The isolation of endothelial cells by an immunobead protocol has been reported elsewhere (41). Briefly, mouse lungs were digested in collagenase, filtered through 100- $\mu$ m cell strainers, centrifuged, and washed twice with medium. Cell suspensions were incubated with a monoclonal antibody (rat anti-mouse) against platelet endothelial cell adhesion molecule 1 for 30 min at 4°C. The cells were washed twice with buffer to remove unbound antibody and were resuspended in a binding buffer containing washed magnetic beads coated with sheep anti-rat immunoglobulin G. Attached cells were washed four to five times in cell culture medium and then were digested with trypsin-EDTA to detach the beads. Bead-free cells were centrifuged and resuspended for culture. After two passages, the cells were incubated with fluorescently labeled diacylated low-density lipoprotein, which is taken up only by endothelial cells and macrophages and sorted to homogeneity by fluorescence-activated cell sorting.

**Cell culture and treatments.** The MLEC were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 6.32 g/liter HEPES, and 3.3 ml of endothelial cell growth supplements in humidified incubators at 37°C. For adenoviral infections, the cells were grown to 30% confluence and changed to serum-free medium containing 10<sup>6</sup> PFU/ml of an adenoviral vector inserted with Hu-FLIP<sub>L</sub>, *lacZ*, or *crmA*. Infected cells were incubated for 3 h and then restored to normal Dulbecco's modified Eagle's medium containing 10% fetal calf serum for an additional 2 days of incubation. For hypoxia treatment, the MLEC were grown to 90% confluence, changed to fresh medium, and transferred to an anaerobic chamber (COY Laboratory Products, Inc., Ann Arbor, MI) with 95%

N<sub>2</sub>, 5% CO<sub>2</sub> for 24 h. After hypoxia incubation, the cells were restored to 95% air, 5% CO<sub>2</sub> for various reoxygenation intervals.

**siRNA transfection.** A small interfering RNA (siRNA) transfection kit corresponding to Bax (mouse) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). MLEC were transfected with Bax siRNA or the control siRNA 36 h prior to H/R treatment according to the manufacturer's protocol.

**Cell fractionation.** For mitochondrial isolation, at various times following exposure to H/R, MLEC were harvested in 0.05% digitonin in an extraction buffer containing 50 mM HEPES, pH 7.5, 50 mM KCl, 5 mM EGTA, and 2 mM MgCl<sub>2</sub> with protease inhibitors. The cell extracts were spun at 700  $\times$  g for 10 min. The supernatants were transferred to new tubes and then centrifuged again at 14,000  $\times$  g at 4°C for 20 min. The resulting supernatants were removed and the pellets retained for Western blotting.

For total membrane isolation, MLEC were harvested in MB buffer (20 mM HEPES, pH 7.5, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 1 mM EDTA, 1 mM dichlorodiphenyltrichloroethane, 250 mM sucrose) containing protease inhibitors and homogenized in a 1.5-ml Dounce homogenizer. Nuclei and unbroken cells were removed by centrifugation for 10 min at 500  $\times$  g, and the supernatants were centrifuged at 100,000  $\times$  g for 60 min at 4°C. The resulting pellet contained total cellular membranes.

For plasma membrane isolation, the homogenates of the cells in MBS [25 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES), pH 6.5, 0.15 M NaCl] containing 1% Triton X-100 were adjusted to 40% sucrose by the addition of 2 ml of 80% sucrose prepared in MBS and placed at the bottom of an ultracentrifuge tube. A 5 to 30% discontinuous sucrose gradient was formed above (4 ml of 5% sucrose/4 ml of 30% sucrose, both in MBS lacking detergent) and centrifuged at 39,000 rpm for 18 h in an SW41 rotor (Beckman Instruments, Palo Alto, CA). A band at the interface of 5% and 30% sucrose was collected and used for immunoprecipitation and Western blotting. After direct addition of substrate solution (*p*-nitrophenyl phosphate; R&D Systems, Minneapolis, MN), the absorbance at 405 nm was measured.

The Golgi complex was isolated using sucrose density gradient centrifugation, as described elsewhere (12). After washing with phosphate-buffered saline, the cells were harvested in G buffer (10 mM Tris-HCl, 0.25 M sucrose, 2 mM MgCl<sub>2</sub>, pH 7.4) containing 10 mM CaCl<sub>2</sub> and proteinase inhibitors. The cells were disrupted with 20 strokes in a Potter-type homogenizer. The homogenate was centrifuged at 2,500  $\times$  g for 10 min, and the pellet was discarded. The resulting postnuclear supernatant was harvested, and the sucrose concentration was adjusted to a 1.4 M final concentration. This suspension was loaded onto the bottom of an ultracentrifuge tube and overlaid in succession with 1.2 M, 1.0 M, and 0.8 M sucrose in G buffer. Samples were then centrifuged at 95,000  $\times$  g for 2.5 h. Two bands from the top, representing 0.8/1.0 and 1.0/1.2 M interfaces, were carefully removed, diluted with G buffer without sucrose, collected by centrifugation at 80,000  $\times$  g for 30 min, and used for the experiments. Golgi fraction purity was assessed by enzymatic activity and immunoblotting methods as previously described (40).

**Western blot analysis.** Proteins were isolated from the culture of MLEC with radioimmunoprecipitation assay buffer (1 $\times$  phosphate-buffered saline, 1% [vol/vol] Nonidet P-40, 0.5% [wt/vol] sodium deoxycholate, 0.1% [wt/vol] sodium dodecyl sulfate [SDS], 0.1 mg/ml phenylmethylsulfonyl fluoride, 30  $\mu$ l/ml aprotinin, and 1 mM sodium orthovanadate). For immunoprecipitation, 1  $\mu$ g of anti-Fas antibody was added to 500  $\mu$ g of total protein in 500  $\mu$ l, rotated for 2 h at 4°C, incubated with 20  $\mu$ l of beads (protein A-sucrose; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for another 2 h, spun down at 500  $\times$  g, and washed three times with radioimmunoprecipitation assay buffer. Then, 20  $\mu$ l of loading buffer (100 mM Tris-HCl, 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, and 20% glycerol) was added. For SDS-polyacrylamide gel electrophoresis, samples containing equal amounts of protein were boiled in the loading buffer and separated by SDS-polyacrylamide gel electrophoresis, followed by transfer to polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat milk and stained with the primary antibodies for 2 h at the optimal concentrations. After five washes in phosphate-buffered saline with 0.2% Tween 20, the horseradish peroxidase-conjugated secondary antibody was applied and the blot was developed with enhanced chemiluminescence reagents (Amersham Biosciences, Piscataway, NJ).

**LDH release assay.** Lactate dehydrogenase (LDH) release was measured using a commercially available assay (cytotoxicity detection kit; Roche Molecular Biochemicals, Indianapolis, IN). After gentle agitation, 200  $\mu$ l of medium was removed at different times to be used for the assay. The samples were incubated (30 min) with buffer containing NAD<sup>+</sup>, lactate, and tetrazolium. LDH converts lactate to pyruvate, generating NADH. The NADH then reduces tetrazolium (yellow) to formazan (red), which was detected by absorption at 490 nm.

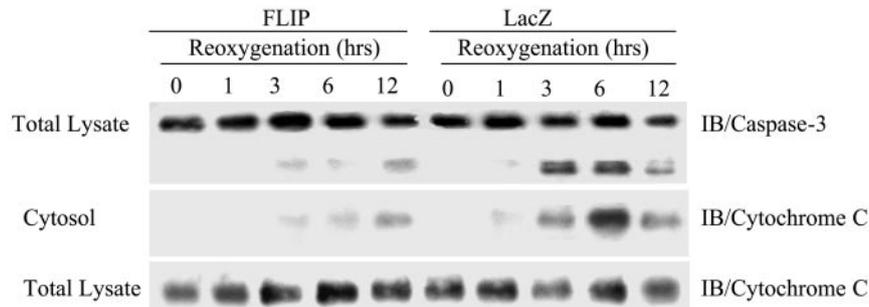


FIG. 1. FLIP inhibits H/R-induced apoptosis in MLEC associated with caspase 3 activation and cytochrome *c* release. MLEC at 30% confluence were cultured in serum-free media in the presence of  $10^6$  CPU/ml of adeno-*FLIP* or adeno-*lacZ* for 3 h and then restored to normal medium. Two days later, the cells were exposed to hypoxia (95%  $N_2$ , 5%  $CO_2$ ) for 24 h and then restored to normoxic culture conditions (95% air, 5%  $CO_2$ ). At the indicated reoxygenation times, total lysates or cytosolic fractions were prepared as described in Materials and Methods. The fractions were subjected to immunoblotting (IB) to detect caspase 3 or cytochrome *c*.

**Statistical analysis.** All values are expressed as means  $\pm$  standard errors. Statistical significance was determined by the Student *t* test, and a value of  $P < 0.05$  was considered significant.

## RESULTS

**FLIP inhibits H/R-induced apoptosis in MLEC associated with caspase 3 activation and cytochrome *c* release.** The effect of FLIP protein on apoptosis was examined in MLEC subjected to H/R treatment. MLEC were infected with adenovirus containing *lacZ* or Hu-*FLIP<sub>L</sub>* (FLIP) for 2 days, exposed to hypoxia (95%  $N_2$ , 5%  $CO_2$ ) for 24 h, and then restored to normoxic culture conditions (95% air, 5%  $CO_2$ ) for various reoxygenation intervals. In *lacZ*-infected controls, H/R treatment caused the manifestation of molecular markers of apoptosis, including caspase 3 activation and appearance of cytochrome *c* in the cytosolic fraction, within 3 to 12 h of reoxygenation (Fig. 1). The activation of caspase 3, as well as cytochrome *c* release as a consequence of H/R treatment, was dramatically reduced in MLEC infected with *FLIP* during 3 to 12 h of reoxygenation (Fig. 1). Thus, FLIP inhibited the appearance of apoptotic markers associated with H/R-induced cell death.

**FLIP inhibits Bax activation and translocation.** H/R treatment caused significant cell death in MLEC as evidenced by LDH release assays (41). We observed that Bax plays a critical role in H/R-induced death in MLEC. MLEC were transfected with Bax siRNA (mouse specific) for 36 h, exposed to hypoxia for 24 h, and then restored to normoxic culture conditions for various reoxygenation intervals. Transfection with Bax siRNA reduced apparent LDH release as a consequence of H/R treatment compared with the control siRNA-transfected cells (Fig. 2A).

We then tested whether FLIP expression can inhibit the intrinsic apoptotic pathway associated with Bax activation. MLEC were infected with adenovirus containing *lacZ* or *FLIP* for 2 days, exposed to hypoxia for 24 h, and then restored to normoxic culture conditions for various reoxygenation intervals. After H/R treatment, cell lysates were immunoprecipitated with the anti-Bax monoclonal antibody (6A7) that specifically recognizes the conformational change in Bax protein associated with its activation (42), followed by immunoblotting to detect Bax. H/R treatment caused an increase in the acti-

vated form of Bax in control (*lacZ*) infected MLEC by 3 h postreoxygenation, which was dramatically inhibited in *FLIP*-infected MLEC (Fig. 2B). We examined whether FLIP may also block Bax mitochondrial translocation. In identical experiments, MLEC infected with *lacZ* or *FLIP* were subjected to H/R treatment and further separated into cytosolic and mitochondrial fractions. H/R treatment of MLEC resulted in the appearance of activated Bax in the mitochondrial fraction. Overexpression of FLIP significantly decreased the translocation of activated Bax to the mitochondria relative to *lacZ*-infected controls (Fig. 2C). In identical experiments, lysates from MLEC subjected to H/R were immunoprecipitated with the anti-Bax monoclonal antibody (6A7) followed by immunoblotting with anti-Bid to examine the association between Bid and activated Bax. In *FLIP*-infected MLEC, dramatically less Bid coimmunoprecipitated in association with activated Bax at 3 h postreoxygenation relative to *lacZ*-infected controls (Fig. 3A). Experiments were performed to determine whether the inhibition of Bax activation by FLIP directly involved downregulation of the caspase 8/Bid pathway. MLEC derived from Bid<sup>-/-</sup> mice were infected with *FLIP* or *lacZ* and subjected to H/R treatments. Cell lysates were immunoprecipitated with the anti-Bax monoclonal antibody (6A7). Surprisingly FLIP inhibited Bax activation in Bid<sup>-/-</sup> MLEC relative to *lacZ*-infected controls subjected to H/R treatment (Fig. 3B), as observed for wild-type MLEC (Fig. 2B). FLIP also protected against H/R-induced cell death in Bid<sup>-/-</sup> MLEC (Fig. 3C) as efficiently as in wild-type MLEC, as previously described (41). Taken together, these results indicate that FLIP inhibits the Bax-dependent (intrinsic) apoptotic pathway by a mechanism independent of the caspase 8/Bid pathway.

**FLIP blocks the H/R-mediated activation of PKC, whose inactive form associates with and inactivates Bax.** We examined alternative mechanisms by which FLIP may inhibit intrinsic apoptosis. Recently, PKC has been implicated as a regulator of many processes associated with apoptosis (7, 34, 36). Therefore, we examined the role of PKC in FLIP-mediated protection against H/R. MLEC were infected with adenovirus containing *lacZ* or *FLIP* for 2 days, exposed to hypoxia for 24 h, and then restored to normoxic culture conditions for various reoxygenation intervals. After H/R treatment, MLEC were separated into cytosolic and membrane fractions to assay

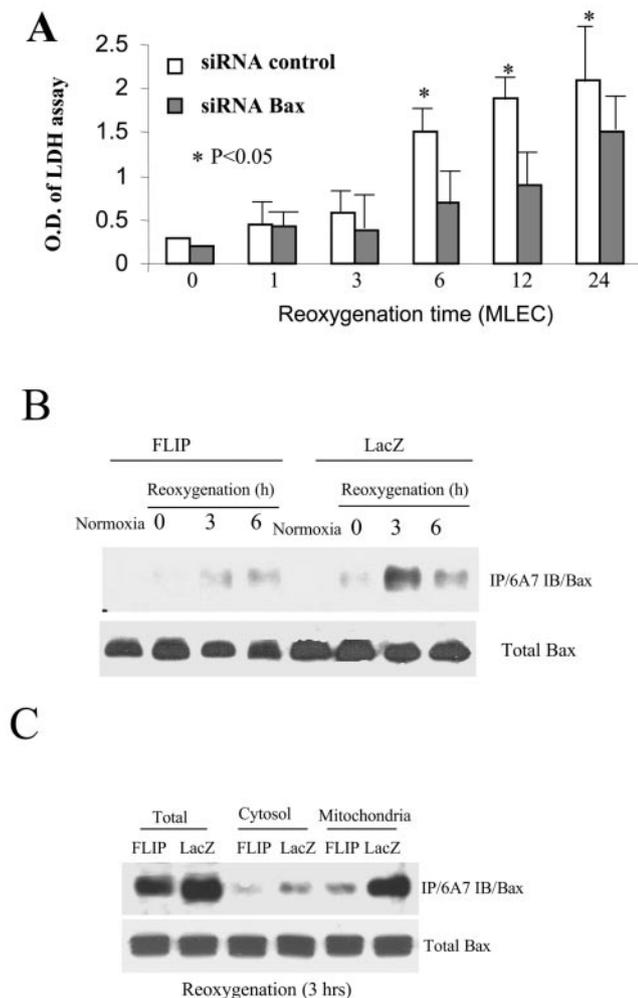


FIG. 2. FLIP inhibits Bax activation induced by hypoxia/reoxygenation in MLEC. MLEC were transfected with Bax siRNA or control siRNA for 36 h, exposed to hypoxia (95% N<sub>2</sub>, 5% CO<sub>2</sub>) for 24 h, and then restored to normal culture conditions (95% air, 5% CO<sub>2</sub>). At the indicated reoxygenation time, 200  $\mu$ l of supernatant medium was removed for LDH assays as described in Materials and Methods (A). MLEC at 30% confluence were cultured in serum-free media in the presence of 10<sup>6</sup> CPU/ml of adeno-*FLIP* or adeno-*lacZ* for 3 h and then restored to complete growth medium. Two days later, the cells were exposed to hypoxia (95% N<sub>2</sub>, 5% CO<sub>2</sub>) for 24 h and then restored to normal culture conditions (95% air, 5% CO<sub>2</sub>) for the indicated reoxygenation times. Total lysates (B) or fractions consisting of cytosol or mitochondria (C) were subjected to immunoprecipitation with antibody 6A7 to detect Bax (immunoblotting [IB]). O.D., optical density.

the expression and activation of PKC. As shown in Fig. 4A, H/R treatment induced PKC expression as well as translocation from the cytosol to the plasma membrane. The expression of FLIP inhibited the expression of PKC during H/R treatment and blocked the translocation of PKC from the cytoplasm to the membrane. Similar results were obtained with an anti-PKC $\alpha$ -specific antibody (data not shown). A series of additional PKC isoforms were tested, including  $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ ,  $\eta$ ,  $\zeta$ , and  $\lambda$ . Of these, only PKC $\zeta$  was strongly activated by H/R treatment (Fig. 4B). FLIP expression suppressed the activation and membrane translocation of PKC $\zeta$  (Fig. 4B). Under normal

conditions, PKC associates with Bax in MLEC. A reduced level of PKC associated with Bax was detected after 24 h of hypoxia, and the level declined further during the first 6 h of reoxygenation (Fig. 4C). The activation of Bax reciprocally increased during the first 3 h of reoxygenation, suggesting that PKC binding to Bax might block Bax apoptotic activity (18).

Next, we examined the effect of FLIP on the association between PKC and Bax. Anti-PKC and anti-Bax immunoprecipitation revealed that FLIP stabilized the association between PKC and Bax in MLEC subjected to H/R during the first 6 h of reoxygenation (Fig. 4D). In a fashion similar to FLIP expression, the inclusion of the PKC inhibitor GF109203X into the hypoxic phase resulted in the stabilization of the association between PKC and Bax during 12 h of reoxygenation. These results indicated that FLIP inhibits PKC activation induced by H/R. The results also suggest that FLIP stabilizes an association between inactivated PKC and Bax protein, which blocks Bax activation and its downstream signaling to the intrinsic apoptotic pathway.

**FLIP inhibits DISC formation in the plasma membrane.** The relative contribution of extrinsic apoptotic pathways to H/R-induced cell death has been examined previously (40, 41). Since FLIP is a known inhibitor of caspase 8, we examined the involvement of caspase 8 processing in H/R-induced cell death. MLEC were infected with adenovirus containing *lacZ* or the caspase 8 inhibitor *crmA* for 2 days, exposed to hypoxia for 24 h, and then restored to normoxic culture conditions for various reoxygenation intervals. Inhibition of caspase 8 by CrmA expression caused a significant decrease in H/R-induced cell death during 3 to 12 h of reoxygenation compared to *lacZ*-infected controls (Fig. 5A).

Next, the effect of FLIP protein on DISC formation was examined in MLEC subjected to H/R treatment. MLEC were infected with adenovirus containing *lacZ* or Hu-*FLIP<sub>L</sub>* (FLIP) for 2 days, exposed to hypoxia (95% N<sub>2</sub>, 5% CO<sub>2</sub>) for 24 h, and then restored to normoxic culture conditions (95% air, 5% CO<sub>2</sub>) for various reoxygenation intervals. As shown in Fig. 5B, hypoxia treatment caused an elevation of total DISC formation, as detected by immunoprecipitation with anti-Fas followed by immunoblotting with caspase 8, which persisted at least 3 h postreoxygenation and began to decline at 6 h postreoxygenation. Surprisingly, transfection of MLEC with *FLIP* resulted in marked inhibition of the DISC formation during the first 3 h of reoxygenation relative to *lacZ*-infected controls.

To examine the effect of FLIP expression on the subcellular localization of the DISC, MLEC infected with *FLIP* or *lacZ* were subjected to H/R treatment and then further separated into Golgi or plasma membrane fractions. MLEC infected with *FLIP* displayed a greater accumulation of the DISC in the Golgi fraction following H/R treatment relative to *lacZ*-infected controls (Fig. 5B). In contrast, MLEC infected with *FLIP* displayed a diminished accumulation of the DISC in the plasma membrane fraction following H/R treatment relative to *lacZ*-infected controls (Fig. 5C).

**FLIP stabilizes Bcl-X<sub>L</sub> protein after reoxygenation.** Bcl-X<sub>L</sub> can block mitochondrial release of cytochrome *c* induced by Bax or Bid. Bcl-X<sub>L</sub> was expressed in normal MLEC after 24 h of hypoxia and decayed to background levels during the first 6 h of reoxygenation (Fig. 6). Infection of MLEC with *FLIP* stabilized Bcl-X<sub>L</sub> protein expression for at least 12 h postreoxy-

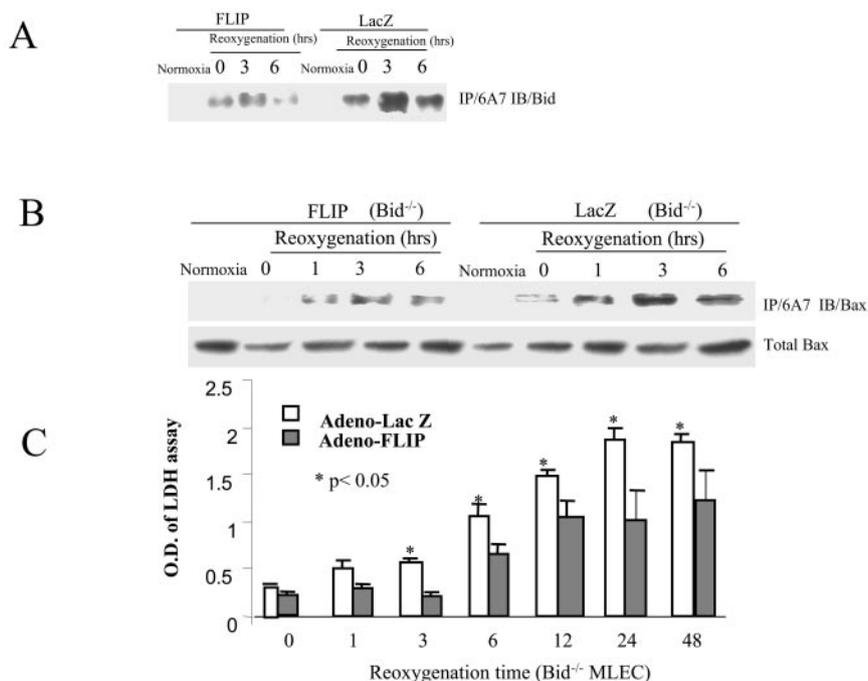


FIG. 3. FLIP blocks the association of Bid and Bax and inhibits Bax-induced cell death in the absence of Bid. Wild-type (A) or Bid knockout (Bid<sup>-/-</sup>) (B) MLEC at 30% confluence were cultured in serum-free media in the presence of 10<sup>6</sup> CPU/ml of adeno-FLIP or adeno-lacZ for 3 h and then restored to normal medium. Two days later, cells were exposed to hypoxia (95% N<sub>2</sub>, 5% CO<sub>2</sub>) for 24 h and then restored to normoxic culture conditions (95% air, 5% CO<sub>2</sub>). At the indicated times, cell lysates were subjected to immunoprecipitation (IP) with antibody 6A7 followed by immunoblotting (IB) to detect Bid (A) or Bax (B). For the toxicity assay, MLEC (Bid<sup>-/-</sup>) were treated with H/R as described above. At the indicated reoxygenation time, 200  $\mu$ l of supernatant medium was removed for LDH assays as described in Materials and Methods (C). The data represent averages of the results from two independent experiments with each sample in triplicate ( $n = 3$ ). Data from adeno-FLIP-infected cells were compared with control (adeno-lacZ-infected) cells at each time point using Student's  $t$  test ( $*P < 0.05$ ). O.D., optical density.

generation relative to the lacZ-infected controls (Fig. 6). These results suggest that another mechanism of FLIP-mediated cytoprotection in MLEC may involve the enhanced expression of Bcl-X<sub>L</sub>, which inhibits Bax- and Bid-induced apoptotic cell death despite the mitochondrial translocation of these species.

## DISCUSSION

Hypoxia, or low oxygen tension, represents a physiologically relevant stress that affects lung and cardiovascular function. Acute hypoxia may cause dilation of the systemic vasculature and constriction of the pulmonary vasculature. Prolonged pulmonary vasoconstriction during sustained hypoxia may lead to the development of pulmonary vascular remodeling, increased vascular resistance, and ultimately, pulmonary hypertension (17). Additionally, cells and tissues exposed to hypoxia during ischemia may sustain damage and cell death upon reoxygenation by restitution of blood flow. This I/R-related injury has long been associated with the enhanced production of reactive oxygen intermediates generated during reoxygenation. Additionally, I/R may cause injury through the recruitment of proinflammatory leukocytes. Endothelial cells represent the first cell type injured by reactive oxygen species generated during I/R (30). In vitro, the mechanisms of endothelial cell death caused by H/R remain poorly understood and may involve both necrotic and apoptotic forms of cell death.

Previously, we presented evidence that H/R stress activated

the Fas-mediated apoptotic pathway in MLEC, especially at early time points (40, 41). In the present model of the extrinsic apoptotic pathway, FADD binds to the receptor Fas, leading to the recruitment of procaspase 8 to form the DISC. The association of caspase 8 with the DISC leads to its autocleavage and activation. Caspase 8 in turn cleaves effector caspases as well as Bid, which stimulates loss of cytochrome *c* from the mitochondria. Recent studies show that the Bid-mediated mitochondrial pathway is critical to ischemic neuronal apoptosis and focal cerebral ischemia in a murine model (23, 43). Interestingly, the FLIP proteins, which effectively resemble catalytically inactive procaspase 8 molecules, act as important regulators of death receptor-induced apoptosis. Increased levels of FLIP can confer protection against Fas-induced apoptosis. Both FLIP<sub>L</sub> and FLIP<sub>S</sub> can be recruited to the DISC, but they function differently. FLIP<sub>S</sub> prevents the initial cleavage step of caspase 8 activation between the p20 and p10 subunits of the caspase homology domain, whereas FLIP<sub>L</sub> inhibits the final cleavage between the prodomain and the p20 subunit of the p43/41 intermediate (16). However, recent reports show that FLIP<sub>L</sub>, when recruited to the DISC, can also potentially promote caspase 8 activation by dimerizing with caspase 8 (33).

Cells can express Fas both intracellularly and at the cell surface. Cytoplasmic Fas localizes in the Golgi apparatus (14), a series of functionally distinct processing compartments that plays a critical role in the biosynthesis and secretion of macromolecules in eukaryotic cells (44). Other

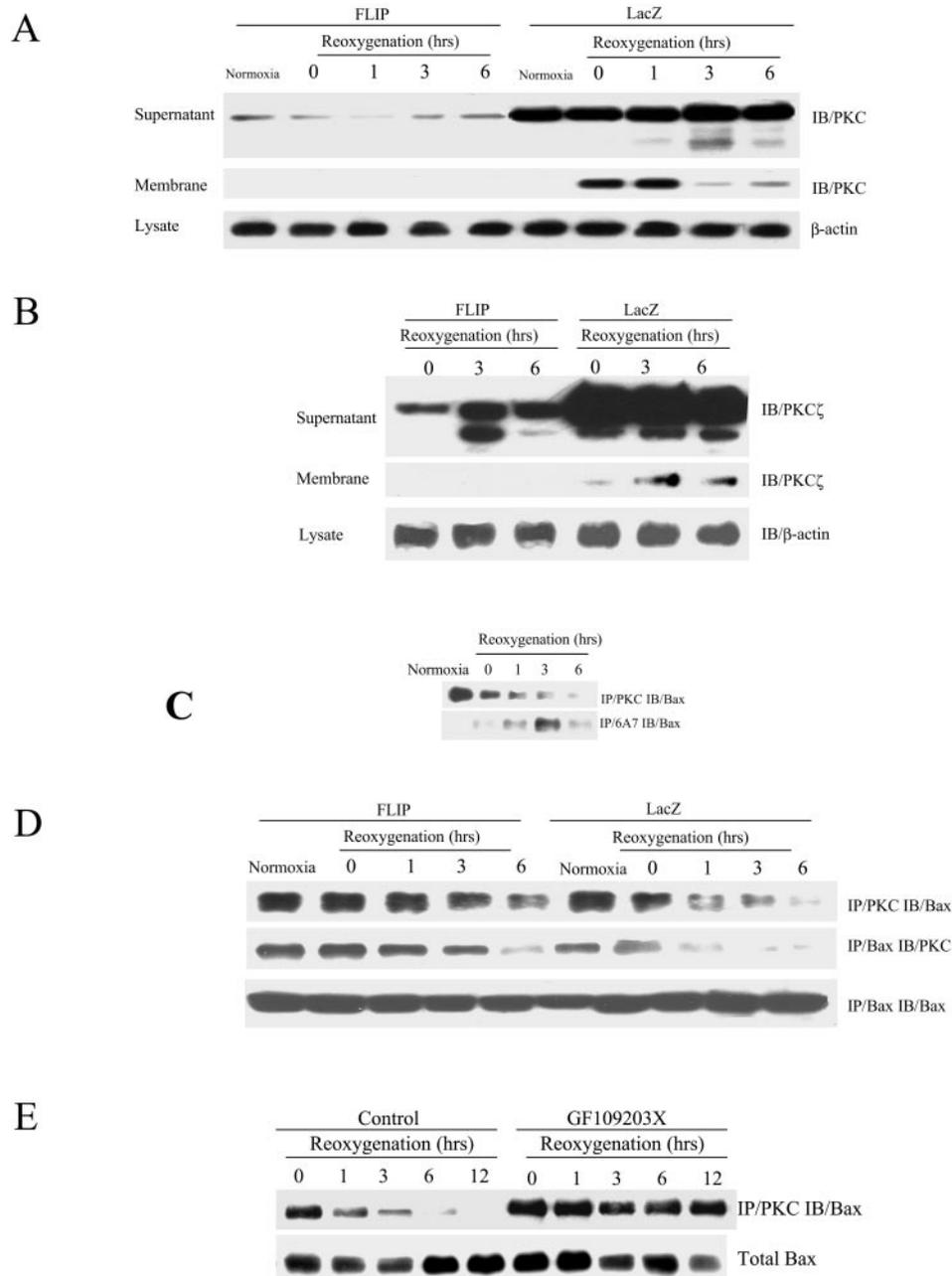


FIG. 4. PKC is inactivated by FLIP and associates with Bax. MLECs at 30% confluence were cultured in serum-free media in the presence of  $10^6$  CPU/ml of adeno-FLIP and adeno-lacZ for 3 h and then restored to normal medium. Two days later, cells were exposed to hypoxia (95%  $N_2$ , 5%  $CO_2$ ) for 24 h and then restored to normal culture conditions (95% air, 5%  $CO_2$ ) for the indicated times. Cell lysates were separated into membrane and supernatant fractions and subjected to immunoblotting (IB) to detect PKC using total PKC antibody (A3) raised against PKC $\alpha$  (A) or  $\zeta$ -specific anti-PKC (B). MLECs grown to 90% confluence were exposed to hypoxia (95%  $N_2$ , 5%  $CO_2$ ) for 24 h and then restored to normal culture conditions (95% air, 5%  $CO_2$ ) for the different periods of time indicated. Cell lysates were subjected to direct immunoprecipitation with PKC (A3) or activated Bax (6A7) antibodies followed by immunoblotting to detect Bax (IB) (C). Total lysates as described for panel A were subjected to direct immunoprecipitation with PKC or Bax followed by immunoblotting to detect Bax or PKC (D). MLECs grown to 90% confluence were exposed to hypoxia (95%  $N_2$ , 5%  $CO_2$ ) for 24 h in the absence or presence of protein kinase C inhibitor GF109203X (400 ng/ml) and then restored to normal culture conditions (95% air, 5%  $CO_2$ ) for the different periods of time indicated. Cell lysates were subjected to direct immunoprecipitation with PKC antibody (A3) followed by immunoblotting to detect Bax (E).

DISC components, such as FADD and caspase 8 proteins may also localize within the Golgi complex for processing and then transfer to the plasma membrane (29, 38, 13). After treatment of MLECs with H/R, we have shown that

the DISC can form within the Golgi complex (Fig. 5B) and translocate to the plasma membrane. Here, we also demonstrate that FLIP<sub>L</sub> may detain the DISC in the Golgi complex (Fig. 5B). We and others have previously shown that other

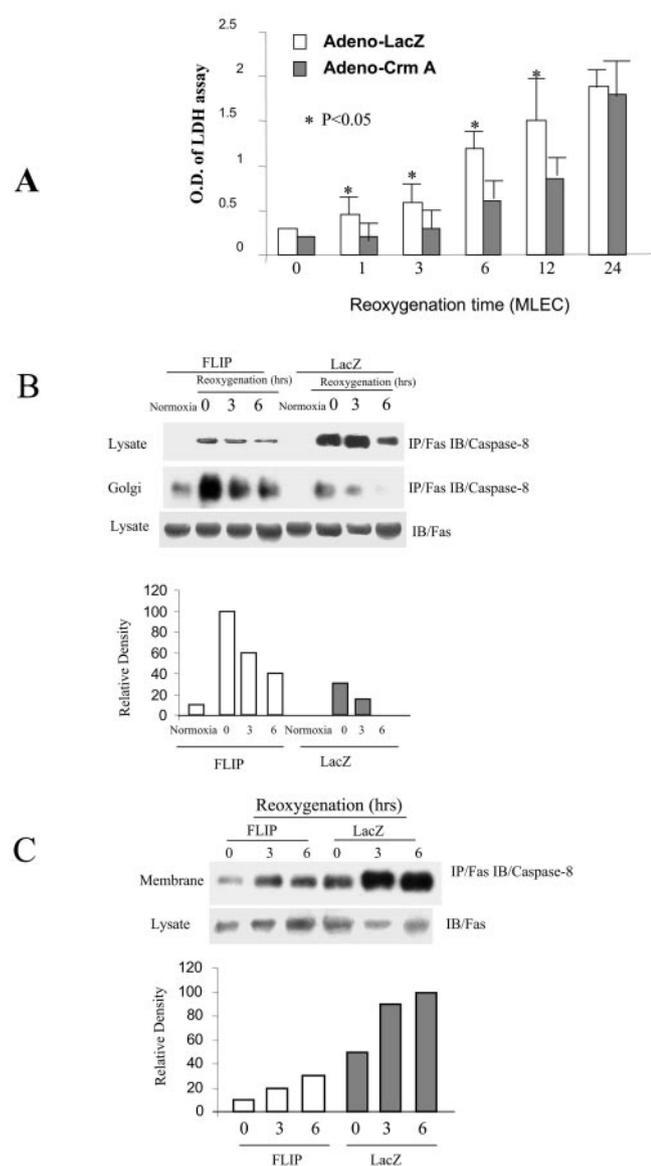


FIG. 5. FLIP inhibits DISC formation in the plasma membrane and retains the DISC in the Golgi apparatus. MLEC at 30% confluence were cultured in serum-free media in the presence of  $10^6$  CPU/ml of adeno-*crmA* or adeno-*lacZ* for 3 h and then restored to normal medium. Two days later, the cells were exposed to hypoxia (95%  $N_2$ , 5%  $CO_2$ ) for 24 h and then restored to normoxic culture conditions (95% air, 5%  $CO_2$ ). At the indicated reoxygenation time, 200  $\mu$ l of supernatant medium was removed for LDH assays as described in Materials and Methods (A). MLEC at 30% confluence were cultured in serum-free media in the presence of  $10^6$  CPU/ml of adeno-*FLIP* or adeno-*lacZ* for 3 h and then restored to normal medium. Two days later, the cells were exposed to hypoxia (95%  $N_2$ , 5%  $CO_2$ ) for 24 h and then restored to normoxic culture conditions (95% air, 5%  $CO_2$ ). At the indicated times, total lysates were prepared (B) and further fractionated into the plasma membrane (C) or Golgi complex (B) as described in Materials and Methods. Each fraction was subjected to immunoprecipitation (IP) with anti-FAS followed by immunoblotting (IB) to detect caspase 8. O.D., optical density.

apoptosis-related molecules, such as FAP-1 (13) or Bcl- $X_L$  (40), also block DISC translocation.

H/R treatment enhanced Bax translocation in MLEC without significantly changing total Bax protein levels (41). The

modulation of Bax translocation during H/R is not understood. Bax is subject to inhibition by Bcl-2 and/or Bcl- $X_L$ , which block cytochrome *c* release from the mitochondria (28). On the other hand, Bid promotes Bax-dependent apoptosis by facilitating a conformational change in Bax associated with its activation (8, 39). Here, the results indicate that FLIP may block Bax activation and its translocation (Fig. 2). The mechanism for this inhibition apparently occurs independently of cross-regulation from Bid, since the effect was sustained in Bid $^{-/-}$  MLEC (Fig. 3B).

PKC comprises a family of Ser/Thr kinases which promote cell survival and protect against cell death. The PKC family consists of 12 closely related isozymes that can be divided into three groups determined by their requirements for activation, the conventional PKCs ( $\alpha$ ,  $\beta_1$ ,  $\beta_2$ , and  $\gamma$ ), the novel PKCs ( $\delta$ ,  $\epsilon$ ,  $\phi$ , and  $\eta$ ), and the atypical PKCs ( $\zeta$ ,  $\lambda$ , and  $\tau$ ) (21). Various studies have implicated the involvement of PKC in the activation of phosphatidylinositol 3-kinase (35) and the mitogen-activated protein kinase pathway (10, 11, 26, 35) as well as inactivation of the Bcl- $X_L$ /Bcl-2-associated death promoter during the inhibition of FAS-mediated signaling (36). PKC upregulates FLIP (34), downregulates caspase 8, and regulates FADD recruitment and DISC formation (7). An investigation into the biological significance of the association of PKC with Bax provided the first evidence of an inverse relationship between the endogenous levels of PKC and the apoptotic effects of phorbol esters (19).

PKC associates with Bax in MLEC under normal culture conditions, which may inhibit the Bax-dependent apoptotic pathway (Fig. 4C and 7).

It has been reported that PKC can be induced by hypoxia (3, 6, 22). Our data show that the expression and plasma membrane translocation of PKC is activated by hypoxia in MLEC, which preferentially involved the  $\alpha$  and  $\zeta$  isoforms (Fig. 4A and B). The overexpression of FLIP inhibits the expression and plasma membrane translocation of PKC $\alpha/\zeta$  under H/R. The inactive forms of PKC, which potentially include PKC $\alpha$  and  $\zeta$  (Fig. 4A and B) but not the other isoforms tested ( $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ ,  $\eta$ ,  $\lambda$ ) (data not shown), bind to Bax to suppress Bax activity induced by H/R. Currently, we do not understand the mechanism by which FLIP inhibits PKC translocation, and this warrants further investigation.

Overexpression of FLIP also stabilizes Bcl- $X_L$  (Fig. 6), which is downregulated by H/R. In our laboratory, we have previously found that Bcl- $X_L$  inhibits both apoptotic pathways (40). Bcl- $X_L$  inhibits Bax activation and Bax redistribution. Furthermore, Bcl- $X_L$  inactivates caspase 8 by disrupting DISC formation in the plasma membrane, Golgi apparatus, and nucleus, while diverting the DISC to the mitochondria where caspase 8 is inactivated. Bcl- $X_L$  breaks the physical association of Fas and caspase 8 with GRASP65, a Golgi apparatus-related protein, suggesting that Bcl- $X_L$  decreases the transfer of Fas and caspase 8 to the plasma membrane by the Golgi component, while promoting its accumulation in the mitochondria. We currently do not understand the mechanism by which FLIP may regulate Bcl- $X_L$ .

The extracellular signal-regulated kinases ERK1/2 respond to stimulation by growth-related signals and play a key role in the regulation of many cellular processes, such as cell growth and proliferation, differentiation, and apoptosis. Hypoxia can

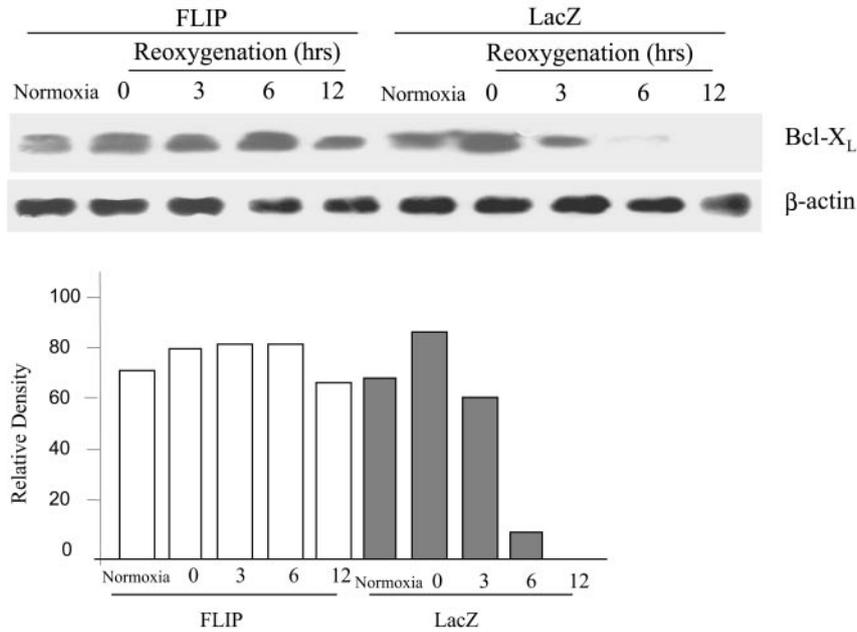


FIG. 6. FLIP upregulates Bcl-X<sub>L</sub> in MLEC. MLEC at 30% confluence were cultured in serum-free media in the presence of 10<sup>6</sup> CPU/ml of adeno-FLIP or adeno-lacZ for 3 h and then restored to normal medium. Two days later, cells were exposed to hypoxia (95% N<sub>2</sub>, 5% CO<sub>2</sub>) for 24 h and then restored to normal culture conditions (95% air, 5% CO<sub>2</sub>). At the indicated reoxygenation times, cell lysates were subjected to Western blot analysis to detect Bcl-X<sub>L</sub>.

induce PKC-dependent ERK1/2 activation (6). The ERK1/2 signaling pathway can induce Bcl-2 and Bcl-X<sub>L</sub> expression and inhibit caspase 3 activation (4). This pathway is also involved in modulating the activity of antiapoptotic molecules, including the phosphorylation and inactivation of Bcl-2 (31). In activated human T lymphocytes, Fas-recruited FLIP activated the

ERK1/2 signaling pathway and the NF-κB pathway through interactions with tumor necrosis factor receptor-associated factors 1 and 2 as well as with the kinases RIP and Raf-1 (15). In our experimental model, FLIP activated the ERK1/2 signaling pathway during H/R (data not shown), which is consistent with previous studies. However, FLIP also inhibits PKC activation,

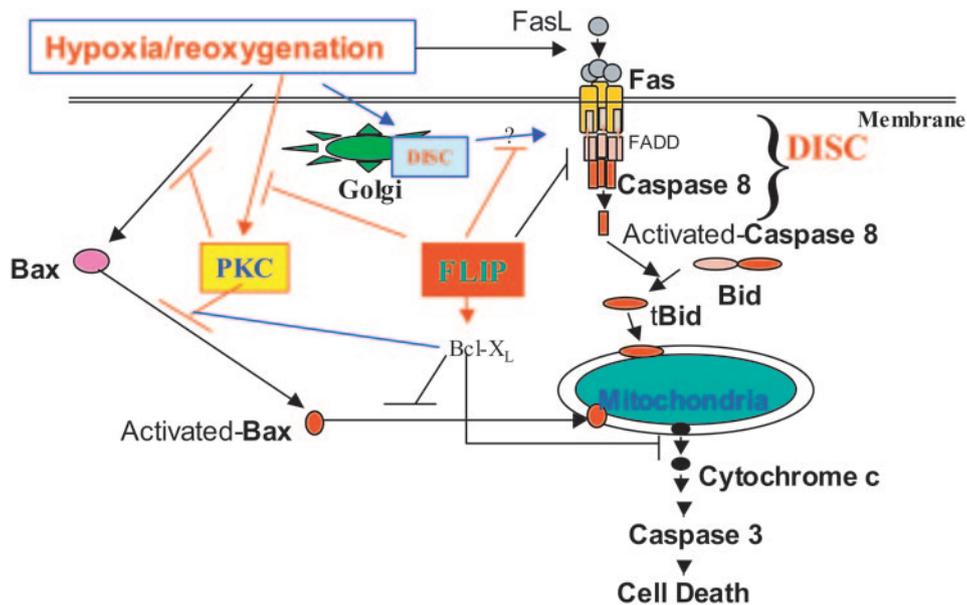


FIG. 7. Pathways of H/R-induced cell death. The diagram depicts the pathways by which H/R stress triggers cell death in MLEC. FLIP inhibited H/R-induced cell death by decreasing the formation of the DISC in the plasma membrane and retaining the DISC within the Golgi apparatus. FLIP also inhibited PKC activation induced by H/R. The inactive form of PKC inhibits Bax activity through their association.

suggesting that the activation of ERK1/2 by FLIP does not depend on PKC activities. Furthermore, FLIP expression did not have an apparent effect on cell growth over 3 days (data not shown).

In summary, H/R induces mouse lung endothelial cell death by activating both extrinsic (Fas/caspase 8/Bid-dependent) and intrinsic (Bax/mitochondrion-dependent) apoptotic signaling pathways (Fig. 7). FLIP, an inhibitor of caspase 8, can inhibit both apoptotic pathways in the context of H/R stress. In addition to inhibiting the recruitment of caspase 8 into the DISC by decreasing DISC formation in the plasma membrane, FLIP blocks the transfer of the DISC formed in the Golgi apparatus to the plasma membrane. FLIP expression also inhibits Bax activation and Bax-induced apoptotic cell death by promoting the association of the inactive form of PKC to Bax, which inactivates Bax.

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