Calcium/Calmodulin-dependent Protein Kinase II Regulation of c-FLIP Expression and Phosphorylation in Modulation of Fas-mediated Signaling in Malignant Glioma Cells

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Fas, upon cross-linking with Fas ligand (FasL) or Fas agonistic antibody, transduces apoptotic yet also proliferative signals, which have been implicated in tumor pathogenesis. In this study, we investigated the molecular mechanisms that control Fas-mediated signaling in glioma cells. Fas agonistic antibody, CH-11, induced apoptosis in sensitive glioma cells through caspase-8 recruitment to the Fas-mediated death-inducing signaling complex (DISC) where caspase-8 was cleaved to initiate apoptosis through a systematic cleavage of downstream substrates. In contrast, CH-11 stimulated cell growth in resistant glioma cells through recruitment of c-FLIP (cellular Fas-associated death domain (FADD)-like interleukin-1β-converting enzyme (FLICE)-inhibitory protein) to the Fas-mediated DISC. Three isoforms of long form c-FLIP were detected in glioma cells, but only the phosphorylated isoform was recruited to and cleaved into a p43 intermediate form in the Fas-mediated DISC in resistant cells. Calcium/calmodulin-dependent protein kinase II (CaMK II) activity was up-regulated in resistant cells. Treatment of resistant cells with the CaMK II inhibitor KN-93 inhibited CaMK II activity, reduced c-FLIP expression, inhibited c-FLIP phosphorylation, and rescued CH-11 sensitivity. Transfection of CaMK II cDNA in sensitive cells rendered them resistant to CH-11. These results indicated that CaMK II regulates c-FLIP expression and phosphorylation, thus modulating Fas-mediated signaling in glioma cells.

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Fas (CD95 or APO-1) induces apoptosis upon stimulation with FasL (CD95L or APO-1L) or an agonistic Fas antibody such as CH-11 (1–4). Fas is a type I transmembrane protein that has multiple cysteine-rich repeats in the extracellular domain and an intracellular motif termed a death domain (DD). Binding of FasL or CH-11 to the Fas extracellular domain induces trimerization of Fas, resulting in the recruitment of the intracellular adapter FADD (5, 6). FADD has a carboxy-terminal DD and an amino-terminal death effector domain (DED). Through its DED, FADD recruits the DED-containing apoptosis-initiating proteases caspase-8 (7, 8) and caspase-10 (9–11) to the Fas receptor to assemble a DISC (12). In the DISC, caspase-8 is cleaved through autoproteolysis of caspase-8 molecules in close proximity (13). Active caspase-8 subunits are released into the cytoplasm to cleave downstream effector caspases such as caspase-3 (14), which subsequently cleaves its substrates such as DNA fragmentation factor 45 (DFF45) (15), to execute programmed cell death.

Recently, the recruitment of other DED-containing proteins to the Fas-mediated DISC, which has been described, modulates DISC functions. These include a family of virus-encoded proteins referred to as v-FLIP (16, 17). v-FLIP contains two DEDs that can bind to the Fas/FADD complex to inhibit Fas-mediated apoptosis by interfering with the recruitment of caspase-8 to the DISC. A mammalian cellular homolog of v-FLIP is termed c-FLIP (18). CASH (19), CASPER (20), CLARP (21), FLAME1 (22), I-FLICE (23), MRIT (24), and Usurpin (25). These studies, however, have generated controversy as to the functions of c-FLIP in apoptosis. Some groups have described it as pro-apoptotic (19–21, 24), whereas others as anti-apoptotic (18, 22, 25). Recent analysis of Fas-mediated DISC in c-FLIP-transfected BJAB cells has shown that c-FLIP proteins are recruited to the Fas-mediated DISC to inhibit caspase-8 cleavage (26, 27), which supports the role of c-FLIP as an anti-apoptotic molecule.

The c-FLIP gene is composed of 13 exons that are clustered within ~200 kilobases within the caspase-8 and caspase-10 genes on human chromosome 2q33 to 34 (25, 28). c-FLIP is expressed as four main mRNA splice variants but only two forms of protein in human tissues (18, 20). The short form protein (c-FLIP S, M s ~28) contains two DEDs and is structurally related to v-FLIP; the longer form (c-FLIP L, M l ~55) is structurally similar to caspase-8 and contains two DEDs and a caspase-like domain that lacks catalytic activity (18). c-FLIP L is expressed in many tissues, but c-FLIP S is found mainly in lymphatic tissue (18). Expression of c-FLIP mRNA and proteins is regulated by mitogen-activated protein kinase kinase in T lymphocytes (29) and by phosphatidylinositol 3-kinase in tumor cells (30). Here we show calcium/calmodulin-dependent protein kinase II (CaMK II) regulates c-FLIP expression and phosphorylation in malignant glioma cells.

Many tumor cells express Fas yet are resistant to Fas-medi-
ated apoptosis (31, 32). An earlier observation, that c-FLIP is overexpressed in human melanomas, suggests that c-FLIP may inhibit Fas-mediated apoptosis in these tumors (33). Recent studies in vivo have shown that high c-FLIP expression in transplanted tumor cells promotes tumor growth and facilitates tumor immune escape (34, 35), which further strengthens the concept that c-FLIP up-regulation is implicated in tumor pathogenesis. To investigate the molecular mechanisms in c-FLIP-mediated regulation of Fas signaling, we examined Fas-mediated DISC and its modulation in glioma cells and showed that c-FLIP regulates Fas signaling through its recruitment to the Fas-mediated DISC. Furthermore, we have demonstrated that CaMK II regulates c-FLIP expression and phosphorylation, thus modulating Fas-mediated signaling in malignant glioma cells.

EXPERIMENTAL PROCEDURES

**Human Glioma Cell Lines, Antibodies, and Reagents**—The human malignant glioma cell lines LN-18, LN-215, LN-464, and LN-443 (gifts from N. De Tribolet, Lausanne, Switzerland) and U343MG and T98G (American Type Culture Collection) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (In-vitrogen). Primary monoclonal antibodies used in the study included anti-human Fas clone 13, FADD, and CaMK II (Transduction Laboratories, Lexington, KY), Fas CH-11, caspase-8, and caspase-10 (Medical & Biological Laboratories, Nagoya, Japan), DFF45 (StressGen, Victoria, BC, Canada), and c-FLIP NF6 clone (26). Primary polyclonal rabbit antibodies included anti-human c-FLIP-3, c-FLIP, and ERK1/2 (StressGen, Victoria, BC, Canada), phosphotheorein (Research Diagnostics, Inc. Flanders, NJ), and PED serum (36). Secondary antibodies included HRP-conjugated goat anti-mouse IgG2b and IgG1 antibodies (Southern Biotech, Birmingham, AL) and HRP-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). [32P]Orthophosphate (Amersham Biosciences), phosphate-free Dulbecco’s modified Eagle’s medium (Invitrogen), the CaMK II activity assay kit (Upstate Biotechnology, Lake Placid, NY), and [γ-32P]ATP (3000 Ci/mmol) (PerkinElmer Life Sciences) were purchased from the commercial sources. Goat anti-mouse IgM-agarose, KN-93, acid phosphatase, complete protease inhibitor mixture, Triton X-100, CHAPS, and all other chemicals of analytical grade were purchased from Sigma.

**Cell Death Assay and Cleavage of Caspases, DFF45, and c-FLIP**—For cell death, cells were seeded in 96-well plates at 2 × 10⁴ cells/well and treated at 37 °C for 24 h with 1 μg/ml anti-Fas CH-11 or 100 μM KN-93 alone or in combination. Cell death was determined by crystal violet assay (37). For cleavage of caspases, DFF45, and c-FLIP, subconfluent cells were treated with 1 μg/ml CH-11 either in the presence or absence of 100 μM KN-93 at 37 °C for the times indicated. At each time point, cells were lysed in lysis buffer (1% Triton X-100, 150 mM NaCl, 10% glycerol, 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, complete protease inhibitor mixture). After centrifugation at 16,000 × g for 15 min at 4 °C, supernatants were subjected to Western blotting.

**DISC Analysis by Immunoprecipitation**—Immunoprecipitation for DISC analysis was performed according to a modified protocol as we previously reported (38). 5 × 10⁴ cells were stimulated with 1 μg/ml CH-11 (mouse IgM) for 30 min at 37 °C and then lysed for 30 min on ice with lysis buffer. In unstimulated controls, the cells were lysed, and 1 μg/ml of CH-11 was added to 1 ml of cell lysates to immunoprecipitate non-stimulated Fas receptors. The solubilized fraction was immunoprecipitated with 20 μl of goat anti-mouse IgM-agarose overnight at 4 °C and analyzed by Western blotting and two-dimensional PAGE immunoblotting.

**Two-dimensional-PAGE, ³²P-Labeling, and Deyrophorization**—For each sample, 1 × 10⁶ cells were either treated with 100 μM KN-93 for 24 h at 37 °C or left untreated and lysed in 40 mM Tris-HCl, pH 8.0, 1% Triton X-100, 65 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, complete protease inhibitor mixture. After centrifugation, the supernatants were precipitated with acetone. The pellets were dissolved in 9.8 M urea, 6% CHAPS, 0.5% pH 3–10 non-linear IPG buffer (Amersham Biosciences) 65 mM dithiothreitol, applied by rehydration in 100 mM pH 3–10 non-linear IPG buffer and composed with IEF Phor™ System following the manufacturer’s protocol (Amersham Biosciences). The strips were equilibrated with 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 10 mg/ml dithiothreitol and subjected to SDS-PAGE followed by transfer to a nitrocellulose membrane (Bio-Rad) and immunoblotting. For labeling of cellular phosphoproteins, cells were incubated for 1 h in phosphate-free Dulbecco’s modified Eagle’s medium (DMEM) and then labeled for 4 h in phosphate-free DMEM supplemented with 0.5 μCi/ml ³²P. Cells were lysed, and cell extracts were subjected to two-dimensional-PAGE followed by autoradiography and immunoblotting, as described above. For dephosphorylation of cellular phosphoproteins, cell lysates obtained from cells were treated with 0.2 unit/mg acid phosphatase for 3 h at 37 °C and subsequently subjected to two-dimensional PAGE immunoblotting.

**Western Blot and Two-dimensional PAGE Immunoblot**—For Western blot, cell extracts and immunoprecipitated DISC samples were separated through SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with blocking buffer (5% nonfat dry milk) for phosphoprotein detection, dry milk was substituted with 3% bovine serum albumin, Tris-buffered saline (20 mM Tris-HCl, pH 7.5, 500 mM NaCl), 0.5% Tween 20) for 2 h at room temperature and then incubated overnight at 4 °C with the following primary antibodies diluted in blocking buffer: anti-caspase-8, anti-DFF45 and anti-c-FLIP (1:1000), anti-caspase-3 and anti-FLIP (1:5000), anti-caspase-10, anti-Fas clone 13, anti-CaMK II, and anti-ERK1/2 (1:500), anti-phosphotheorein (1:300), anti-FADD (1:250), and anti-c-FLIP NF6 (1:10). The membranes were washed in Tris-buffered saline, 1% Tween 20 and incubated for 2 h at room temperature with the following secondary antibodies diluted in Tris-buffered saline, 1% Tween 20: anti-mouse IgG2b-HRP, anti-mouse IgG1-HRP (1:20,000), and anti-rabbit IgG-HRP (1:5000). The blots were washed and developed by chemiluminescence (Amersham Biosciences).

**CaMK II Activity Assay and CaMK II cDNA Transfection**—CaMK II activity was assayed in cell lysates using CaMK II assay kits following the manufacturer’s protocol (Upstate Biotechnology). For transfection, CaMK II cDNA (Strategene, La Jolla, CA) was subcloned into pcDNA3.1 expression vector using EcoRI I and HindIII restriction sites. Transfection of the pcDNA3.1 expression vector containing CaMK II cDNA into glioma cells was accomplished using the LipofectAMINE method following the manufacturer’s protocol (Invitrogen). After transfection for 48 h, the cells were subjected to cell death assay and Western blot analysis for cleavage of caspases and DFF45.

**RESULTS**

**Caspase-8 Is Recruited to the Fas-mediated DISC to Initiate Apoptosis**—Malignant glioma cells express cell surface Fas and are susceptible to agonistic Fas antibody-induced apoptosis (31). To illustrate the signal events in Fas-mediated apoptosis, we first analyzed the Fas-mediated DISC in sensitive glioma cells. Three glioma cell lines (U343MG, LN-18, T98G) were transfected for 48 h, the cells were subjected to cell death assay and Western blot analysis to examine endogenous expression of the proteins.

Western blots detected one p45 protein band of Fas, one p25 protein band of FADD, four protein bands of caspase-8, and four protein bands of caspase-10 in the Fas-mediated DISC in CH-11 stimulated cells (Fig. 1A). Two caspase-8 precursor proteins (p55 and p53) were detected only in the DISC. Similarly, two caspase-10 precursor proteins (p59 and p41) were detected only in the DISC. For Western blot analysis to examine endogenous expression of the proteins.
ysis of the cell extracts (Fig. 1A), but they were not detected in the Fas-mediated DISC in these CH-11-sensitive cells (Fig. 1A). PED/PEA-15 was reported to inhibit Fas-mediated apoptosis (39, 40). Western blots failed to detect PED/PEA-15 in the Fas-mediated DISC in these sensitive glioma cells (Fig. 1A).

Caspase-8 cleavage occurs in two consecutive steps in Fas-mediated DISC, first-step cleavage to produce p12, p43, and p41 subunits and second-step cleavage to generate prodomain and active p18 and p10 subunits (41) to cleave downstream caspase-3 p32 precursors into large p20 and p17 and small p10 subunits (42). To examine this caspase-8-initiated cascade, we analyzed cell extracts collected from the cells exposed to 1 μg/ml CH-11 to look for cleavage products. Western blots detected caspase-8 first-step cleavage p43 and p41 products within 30 min and second-step cleavage p18 proteins within 60 min after CH-11 stimulation (Fig. 1B). Western blots also detected caspase-3 cleavage p20 and p17 subunits after CH-11 stimulation (Fig. 1B).

Finally, we examined the cell extracts for cleavage products of DFF45, a caspase-3 downstream substrate (15, 43). Two forms of DFF45, the long form DFF45 and the short form DFF35, were endogenously expressed in the glioma cells and proteolytically cleaved upon CH-11 stimulation (Fig. 1B).

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**CaMK II Regulation of c-FLIP Proteins**

**Resistant Glioma Cells**—Many glioma cell lines express cell surface Fas yet are resistant to CH-11 and soluble FasL (31, 37). To investigate molecular mechanisms of this resistance, we analyzed Fas-mediated DISC in three glioma cell lines (LN-215, LN-443, LN-464) that express Fas but are resistant to CH-11 (data not shown). In the cells stimulated with 1 μg/ml CH-11, Western blots detected one p45 Fas band, one p25 FADD band, and four caspase-8 bands, p55 and p53 caspase-8.
precursors and p43 and p41 caspase-8 first-step cleavage products (Fig. 2A). These findings indicated that FADD and caspase-8 are recruited to the Fas-mediated DISC, where caspase-8 completes its first-step cleavage. However, Western blot analysis failed to show caspase-8 second-step cleavage products in these resistant cells treated with CH-11 (Fig. 2B), indicating that caspase-8 second-step cleavage is inhibited in the DISC. Western blots also failed to detect caspase-3 and DFF45 cleavage products (Fig. 2B), which indicated that caspase-8-initiated cascade is inhibited as well in the resistant cells. Neither caspase-10 precursors nor its cleavage products was detected in the Fas-mediated DISC (data not shown) due to the lower levels of caspase-10 expression in these cells, as we previously reported (38).

We further examined the Fas-mediated DISC to look for c-FLIP and PED/PEA-15. The long form p55 c-FLIP_L and the short form p25 c-FLIP_S were endogenously expressed in these glioma cells (Fig. 2A). However, Western blot analysis of the DISC revealed three forms of c-FLIP proteins, a very weak band of p55 c-FLIP_L, a new band of p43 c-FLIP, and a band of p25 c-FLIP_S (Fig. 2A). These findings indicated that c-FLIP_L is recruited to the DISC and cleaved into an intermediate p43 form, as previously reported in c-FLIP transfectants (18, 26, 27). Caspase-8 first-step cleavage occurred in both CH-11-sensitive and -resistant glioma cells (Figs. 1A and 2A), but it appeared that only resistant cells showed simultaneous recruitment of caspase-8 and c-FLIP_L to the DISC, resulting in c-FLIP_L cleavage into a p43 intermediate form (Fig. 2A). To further confirm this, we examined c-FLIP_L cleavage in the sensitive and resistant cell lines. Indeed, Western blots detected p43 intermediate products in the resistant cells (LN-215, LN-443, LN-464) but not in the sensitive cells (U343MG, LN-18, T98G) after exposure to 1 μg/ml CH-11 (Fig. 2C). Western blots also detected PED/PEA-15 in the Fas-mediated DISC (Fig. 2A). Simultaneous detection of caspase-8 first-step cleavage products, c-FLIP, and PED/PEA-15 suggested that both c-FLIP and PED/PEA-15 proteins may be responsible for inhibition of caspase-8 second-step cleavage in the Fas-mediated DISC in the CH-11-resistant glioma cells.

c-FLIP and CaMK II Are Up-regulated in Resistant Glioma Cells—The differential recruitment of c-FLIP and PED/PEA-15 to the Fas-mediated DISC suggests that c-FLIP and PED/PEA-15 proteins may be up-regulated in the resistant glioma cells. Indeed, our earlier work has shown that CaMK II up-regulates PED/PEA-15 expression and phosphorylation and modulates TRAIL (tumor necrosis factor-related apoptosis-inducing ligand)-induced apoptosis in glioma cells (38). We speculated that a similar mechanism might exist in the glioma cells, which regulates c-FLIP expression and function. To test this hypothesis, we first investigated whether treatment of the resistant cells with KN-93, a CaMK II inhibitor, might restore CH-11 sensitivity. LN-215, LN-443, and LN-464 cell lines were treated with 1 μg/ml CH-11 alone or in the presence of 100 μM KN-93 for 24 h. Crystal violet analysis showed a consistent 20% cell growth in the CH-11-stimulated cells (Fig. 3A). Western blots also failed to show caspase-8 second-step cleavage products, indicating that caspase-8 second-step cleavage is inhibited as well in the resistant cells after KN-93 treatment, we speculated that CaMK II might regulate c-FLIP phosphorylation and recruitment to the DISC. To test this, we first compared the c-FLIP protein expression levels in the CH-11-sensitive and resistant glioma cell lines. Western blot analysis showed that both c-FLIP_L and c-FLIP_S were expressed at higher levels in the resistant (LN-215, LN-443, LN-464) than in the sensitive cell lines (U343MG, LN-18, T98G) (Fig. 4A) and that the c-FLIP_L and c-FLIP_S expression levels were markedly reduced in resistant cells after treatment with 100 μM KN-93 for 24 h (Fig. 4A). Next, we examined CaMK II protein expression in the glioma cells and showed that CaMK II protein was highly expressed in CH-11-resistant cells as compared with the sensitive cells (Fig. 4A). Finally, we examined CaMK II activity to determine whether overexpressed CaMK II is biologically functional in glioma cells. CaMK II activity was measured in a total of 10 μg proteins from each of the cell extracts using a specific kinase substrate and a mixture of kinase inhibitors to block the activity of other kinases. CaMK II activity was significantly higher in the resistant cell lines LN-215, LN-443, and LN-464 than in the sensitive cell lines U343MG, LN-18, and T98G (Fig. 4B). Treatment of the resistant cells with 100 μM KN-93 for 24 h markedly reduced CaMK II activity in the resistant cells (Fig. 4B).

CaMK II Regulates c-FLIP.L Phosphorylation and Recruitment to the DISC—CaMK II regulates c-FLIP expression to modulate Fas-mediated apoptosis in glioma cells. Here, we further proposed that CaMK II might regulate c-FLIP phosphorylation and recruitment to the Fas-mediated DISC. To test this, we first subjected the cell extracts to two-dimensional PAGE immunoblots to identify isoforms of the c-FLIP proteins. Two-dimensional PAGE immunoblots detected one c-FLIP_S
Data represent the means ± S.E. of four experiments from three batches of cells and analyzed by Student's t test (*, p < 0.001 between the untreated and resistant cells; #, p < 0.001 between the untreated and KN-93-treated resistant cells).

Fig. 4. c-FLIP and CaMK II protein expression and CaMK II activity. A, c-FLIP and CaMK II protein expression in glioma cell lines. The cell lines are indicated above the panels. CH-11-sensitive (U343MG, LN-18, T98G) and CH-11-resistant (LN-215, LN-443, LN-464) cell lines were analyzed by Western blot (WB) using anti-c-FLIP N6 and CaMK II antibody in which ERK1/2 was used as a loading control. CH-11-resistant cells were also treated with 100 μM KN-93 for 24 h and analyzed on the WB. B, CaMK II activity in glioma cell lines. Cell extracts were obtained from glioma cells either untreated or treated with 100 μM KN-93 for 24 h, and 10 μg of cell extract from each cell line were analyzed for CaMK II activity following the manufacturer's protocol. CaMK II activity was expressed as pmol/min/mg of protein. Data represent the means ± S.E. of four experiments from three batches of cells and analyzed by Student's t test (*, p < 0.001 between the resistant and sensitive cells; #, p < 0.001 between the untreated and KN-93-treated resistant cells).

Treatment of the resistant cells with 100 μM KN-93 for 24 h inhibited CaMK II activity (Fig. 4B) and eliminated the expression of the isoform c-FLIP S, indicating that there is only one isoform of c-FLIP S endogenously expressed in glioma cells. In contrast, however, two-dimensional PAGE blots revealed three isoforms of c-FLIP L, indicated as c-FLIP Lα, c-FLIP Lβ, and c-FLIP Lγ, in both sensitive (U343MG, LN-18) and resistant cell lines (LN-215, LN-443), but isoform c-FLIP Lα was detected only in the resistant cell lines (Fig. 5A).

Fig. 5. Two-dimensional PAGE analysis of c-FLIP isoforms. Isoelectric point (PI) ranges are indicated above the panels. A, two-dimensional PAGE immunoblots. Cell lines and treatments are indicated to the left. Cell extracts from CH-11-sensitive cell lines (U343MG, LN-18) and CH-11-resistant cell lines (LN-215, LN-443) that were untreated or treated with either 100 μM KN-93 for 24 h or 0.2 units/ml acid phosphatase for 3 h were subjected to two-dimensional PAGE immunoblotting using anti-c-FLIP polyclonal antibody. The isoform of c-FLIP Lα, c-FLIP Lβ, or c-FLIP Lγ was indicated as a, b, or c, and c-FLIP S and c-FLIP L were indicated under the panels. B, two-dimensional PAGE autoradiography and immunoblots. 32P-Labeled cell lines are indicated above the panels. The cells were labeled with [32P]orthophosphate for 4 h and then subjected to two-dimensional PAGE autoradiography. The arrows indicate the locations of phosphorylated protein spots c-FLIP L (p55) and c-FLIP S (p25). After two-dimensional (2D) PAGE autoradiography, the same membranes were probed with anti-c-FLIP antibody to identify the c-FLIP proteins. The arrows indicate the same spots identified by 32P autoradiography and anti-c-FLIP antibody immunoblotting. WB, Western blot.

The c-FLIP Lα isoform, which is highly expressed in resistant cell lines, is phosphorylated on threonine residues (Fig. 5B). To test this hypothesis, we first treated cell extracts of resistant cell lines LN-215 and LN-443 with acid phosphatase for 3 h at 37 °C and then subjected the cell extracts to two-dimensional PAGE immunoblots. The results showed that acid phosphatase treatment eliminated the c-FLIP Lα isoform in the resistant cells (Fig. 5A). Next, we labeled the resistant cell lines LN-215 and LN-443 with [32P]orthophosphate and subjected the cell extracts to two-dimensional PAGE that was examined first by autoradiography and then by immunoblotting with anti-c-FLIP antibody. Autoradiography of the two-dimensional PAGE membrane revealed one 32P-labeled spot among the three c-FLIP L isoforms (Fig. 5B) and subsequent immunoblot analysis of the same membrane showed that the 32P-labeled spot was identical to c-FLIP Lα (Fig. 5B). In contrast, similar analysis of autoradiography and immunoblots of two-dimensional PAGE failed to show any phosphorylated spot of the c-FLIP S form (Fig. 5B).

CaMK II is a S/T protein kinase (44). To further confirm c-FLIP Lα phosphorylation, we examined cell extracts from the sensitive U343MG and resistant LN-215 cells on two-dimensional PAGE immunoblots using anti-phosphothreonine antibody. The same membranes were then stripped of primary and secondary antibodies and analyzed using c-FLIP antibody. The c-FLIP Lα spot, detected using c-FLIP antibody, was not phosphorylated by CaMK II, as shown in Fig. 6. Taken together, these studies indicated that c-FLIP Lα is a phosphoprotein and is highly expressed in the resistant glioma cells.

We showed that c-FLIP Lα was recruited to the Fas-mediated...
DISC only in the resistant cells (Fig. 2A), and of three isoforms of c-FLIPL, only c-FLIPLb was expressed in the resistant cells (Figs. 5 and 6). These results suggested that c-FLIPLb, but neither c-FLIPLa nor c-FLIPLb, is recruited to the Fas-mediated DISC. Western blot analysis showed that immediately upon recruitment, c-FLIPL proteins were cleaved into p43 intermediate forms in the DISC (Fig. 2A), and thus, we were not surprised by the fact that two-dimensional PAGE immunoblots using anti-c-FLIP antibody detected p43 intermediate form protein but not c-FLIPL in the CH-11-induced DISC in LN-215 cells (Fig. 6). The same membrane was then analyzed using anti-phosphothreonine antibody, and the results showed that p43 intermediate form protein was phosphorylated (Fig. 6); the results suggested that p43 intermediate forms are generated from the phosphoprotein c-FLIPLb but not from c-FLIPLa or c-FLIPLb.

**CaMK II cDNA Transfection in Sensitive Glioma Cells Resulting in Cell Resistance**—To further define the function of CaMK II in modulation of Fas-mediated apoptosis in tumor cells, we transiently transfected CaMK II cDNA in CH-11-sensitive U343MG and LN-18 cell lines. Crystal violet analysis showed a near 100% cell death (Fig. 7A), and remarkably decreased caspase-8, caspase-3, and DFF45 (Fig. 7B). CaMK II cDNA-transfected cells were further exposed to 1 μg/ml CH-11 in the presence of 100 μM KN-93, and cell death was determined by crystal violet assay. Some of the cells were lysed and then analyzed on Western blot with anti-CaMK II antibody. B, cleavage of caspases and DFF45. The transfected cells were treated with 1 μg/ml CH-11 in the presence or absence of 100 μM KN-93 for 3 h, and cell lysates were examined on Western blot (WB) using anti-caspase-8, caspase-3, and DFF45 antibodies.

**DISCUSSION**

The Fas/FasL-signaling pathway is an essential process in the regulation of programmed cell death and has been implicated in the pathogenesis of immune system diseases and various malignancies (45). Recent studies have provided several lines of evidence to support the role of the Fas-signaling pathway in tumorigenesis of malignant gliomas. Gliomas express both Fas and FasL, and glioma-derived FasL induces apoptosis of Fas-positive T cells that infiltrate tumors, thus facilitating tumor escape from the host immune system (46). FasL stimulates gliomas to produce chemokines that modulate immune responses (47). FasL or the Fas agonistic antibody CH-11 triggers apoptosis in some glioma cells (48) but transduces proliferative signals in others (49). In the present study, we analyzed the Fas-mediated DISC and elucidated the molecular mechanisms that regulate the biological functions of Fas-signaling pathways in human gliomas.

The signal transduction of Fas-mediated apoptosis has been
well characterized in transfectants and lymphocytes (45) but remains to be established in non-transfected solid tumor cells. Here, we showed that Fas-mediated apoptosis in sensitive glioma cells occurs through recruitment of apoptosis-initiating caspase-8 and caspase-10 to the Fas-mediated DISC. Caspase-8 completes its consecutive two-step cleavage in the DISC, resulting in release of its active subunits into the cytoplasm that initiate programmed cell death by subsequent cleavage of downstream effector caspases such as caspase-3 (14, 42) and caspase-3 substrates such as DFF45 (15, 43). However, many glioma cells are resistant to Fas-mediated apoptosis. Analysis of the Fas-mediated DISC provides a new molecular model for Fas-mediated resistance in tumor cells. Apoptosis-initiating caspase-8 is recruited to the Fas-mediated DISC and completes its first-step cleavage, but its second-step cleavage is inhibited in the DISC. Detection of c-FLIP and PED/PEA-15 molecules in the DISC suggests that these DED-containing proteins may inhibit the second-step cleavage of caspase-8 and, thus, prevent Fas-mediated apoptosis in tumor cells.

Recent studies of c-FLIP overexpression have provided two models for c-FLIP modulation of the Fas-mediated DISC (26, 27). In the first model, caspase-8 and c-FLIP<sub>L</sub> are simultaneously recruited to the DISC, where caspase-8 completes its first-step cleavage, whereas c-FLIP<sub>L</sub> is cleaved into a p43 intermediate form. The p43 intermediate c-FLIP<sub>L</sub> remains in the DISC to interrupt caspase-8 second-step cleavage, and, thus inhibit caspase-8-initiated apoptosis. In contrast in another model, c-FLIP<sub>L</sub> is recruited to the DISC to inhibit first-step cleavage of caspase-8. Here, we demonstrated the p43 intermediate form in the Fas-mediated DISC, which supports the c-FLIP<sub>L</sub>-mediated model of regulation of Fas-mediated signaling. However, simultaneous detection of c-FLIP<sub>L</sub> and caspase-8 first-step cleavage in the DISC raises a question about the role of c-FLIP<sub>L</sub> in Fas signaling in glioma cells. It also remains to be clarified why both c-FLIP and PED are simultaneously recruited to the Fas-mediated DISC in glioma cells.

There is now increasing evidence that Fas transduces proliferative signals, but the signaling pathways remain poorly defined (50). Overexpression of c-FLIP<sub>L</sub> has been reported to activate nuclear factor kappa B (NF-κB) through unknown signal pathways (51). On the other hand, c-FLIP<sub>L</sub> transfection up-regulates the proto-oncogene c-Fos in FADD- and caspase-8-dependent manner (52); the results suggest that c-FLIP<sub>L</sub>-mediated cell proliferation occurs through the DISC. Here, we showed that glioma cells proliferate in response to CH-11 stimulation. Further detection of c-FLIP<sub>L</sub> and c-FLIP<sub>P</sub> in the Fas-mediated DISC enhances the concept that c-FLIP<sub>L</sub> proteins are recruited to the DISC to activate its downstream proliferative signals. PED/PEA-15 has also been shown to interfere with FADD and caspase-8 interactions and, thus, prevents DISC formation and subsequent apoptosis induced by FasL (39, 40). PED/PEA-15 is recently reported to regulate Ras-initiated mitogen-activated protein kinase pathways through its DED (53) and, thus, modulate integrin-mediated cell growth, migration, and tumor metastasis (54, 55), but the molecular events that connect the PED/PEA-15-mediated apoptotic signaling pathway to the Ras-initiated mitogen-activated protein kinase pathway remain elusive.

We have further identified three isoforms of c-FLIP<sub>L</sub> in glioma cells. The three isoforms were separated by their difference in their molecular weights and isolectric points on two-dimensional PAGE. A combined two-dimensional PAGE 32P autoradiography and immunoblot analysis further revealed that one of the three isoforms was phosphorylated. The phosphorylated c-FLIP<sub>L</sub> was expressed only in resistant cells, and inhibition of CaMK II activity in the resistant cells eliminated this phosphorylated isoform of c-FLIP<sub>L</sub> and rendered the cells sensitive to CH-11-induced apoptosis. Furthermore, we showed that the p43 intermediate form of c-FLIP<sub>L</sub> was phosphorylated, which suggested that the phosphorylated c-FLIP<sub>L</sub> isoform is recruited to and cleaved into a p43 intermediate form in the Fas-mediated DISC. The p43 form of c-FLIP<sub>P</sub> remains bound in the DISC to inhibit caspase-8-initiated apoptosis, and, thus, switch the glioma cells from apoptosis to proliferation.

CaMK II has recently been implicated in the modulation of microcystin-induced apoptosis (56). Our earlier studies showed that CaMK II regulates PED/PEA-15 phosphorylation and, thus, modulates TRAIL (tumor necrosis factor-related apoptosis-inducing ligand)-induced apoptosis in glioma cells (38). We now provide several lines of evidence that CaMK II up-regulates c-FLIP expression and phosphorylation to modulate Fas-mediated signaling in the same tumor cells. Both c-FLIP<sub>P</sub> and c-FLIP<sub>L</sub> were highly expressed in resistant glioma cells, and treatment of these cells with the CaMK II inhibitor KN-93 reduced the expression of both long and short forms of c-FLIP and rescued the cell sensitivity to Fas-mediated apoptosis. CaMK II protein and biological activity were up-regulated, and KN-93 treatment eliminated the phosphorylated c-FLIP<sub>L</sub> in the resistant cells, suggesting that CaMK II may regulate c-FLIP<sub>L</sub> phosphorylation. Other kinases such as mitogen-activated protein kinase kinase, phosphatidylinositol 3-kinase, and protein kinase C have also been reported to regulate c-FLIP and PED/PEA-15 to modulate apoptosis (29, 30, 37). Therefore, further studies of these kinase-mediated signaling pathways will shed some light on the complex regulatory mechanisms that control tumor cell death and proliferation.

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