Journal of Cellular Biochemistry 103:788–799 (2008)

Calmodulin Binding to the Fas-Mediated Death-Inducing Signaling Complex in Cholangiocarcinoma Cells

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Abstract We have previously demonstrated that the antagonists of calmodulin (CaM) induce apoptosis of cholangiocarcinoma cells partially through Fas-mediated apoptosis pathways. Recently, CaM has been shown to bind to Fas, which is regulated during Fas or CaM antagonist-mediated apoptosis in Jurkat cells and osteoclasts. Accordingly, the present studies were designed to determine whether Fas interacts with CaM in cholangiocarcinoma cells and to elucidate its role in regulating Fas-mediated apoptosis. CaM bound to Fas in cholangiocarcinoma cells. CaM was identified in the Fas-mediated death inducing signaling complex (DISC). The amount of CaM recruited into the DISC was increased after Fas-stimulation, a finding confirmed by immunofluorescent analysis that demonstrated increased membrane co-localization of CaM and Fas upon Fas-stimulation. Consistently, increased Fas microaggregates in response to Fas-stimulation were found to bind to CaM. Fas-induced recruitment of CaM into the DISC was inhibited by the Ca^{2+} chelator, EGTA, and the CaM antagonist, trifluoperazine (TFP). TFP decreased DISC-induced cleavage of caspase-8. Further, inhibition of actin polymerization, which has been demonstrated to abolish DISC formation, inhibited the recruitment of CaM into the DISC. These results suggest an important role of CaM in mediating DISC formation, thus regulating Fas-mediated apoptosis in cholangiocarcinoma cells. Characterization of the role of CaM in Fas-mediated DISC formation and apoptosis signaling may provide important insights in the development of novel therapeutic targets for cholangiocarcinoma. J. Cell. Biochem. 103: 788–799, 2008.

Key words: calmodulin; Fas; DISC; apoptosis; cholangiocarcinoma; Fas death receptor

Hepatobiliary cancer is an important health problem in the United States and world wide. Cholangiocarcinoma is the second most common primary malignant tumor of the liver and comprises approximately 20% of all hepatobiliary malignancies in the United States [Ahrendt et al., 2001]. Studies from our group and others have indicated that the regulation of Fas-mediated apoptosis may represent a promising therapeutic avenue for this generally fatal cancer [Pan et al., 1999; Que et al., 1999; Ahn et al., 2002]. Further, we have demonstrated that antagonists of CaM induce apoptosis of cholangiocarcinoma cells in vitro through pathways similar to Fas-mediated apoptosis signaling pathways [Pan et al., 1999; Ahn et al., 2003]. Accordingly, fundamental understanding of the role of CaM in Fas-mediated apoptosis may present new avenues for successful cancer chemotherapy.

The role of Ca^{2+}/CaM in cancer pathogenesis remains unclear and somewhat paradoxical, because Ca^{2+} is critical for both cell division and cell death. Ca^{2+}-dependent pathways regulate synthesis and secretion of several proteases that are involved in the invasive phenotype of tumor cells [Cole and Kohn, 1994]. On the other hand, large and sustained elevations of the cytosolic Ca^{2+} concentration can activate a number of mechanisms that lead to cell death [Nicotera and Orrenius, 1998]. Increased intracellular Ca^{2+} has been associated with Fas-mediated apoptosis in the human B cell line FMO [Oshimi and Miyazaki, 1995], Jurkat cells [Sen et al., 1999], as well as cholangiocarcinoma cells [Ahn et al., 2003]. The ubiquitous Ca^{2+} binding effector protein, CaM, plays an

Grant sponsor: VA Merit Review Award to Jay M. McDonald, M.D.

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Received 29 January 2007; Accepted 11 May 2007
DOI 10.1002/jcb.21447

important role in apoptotic signaling; however, the underlying molecular and cellular mechanisms remain largely unknown. Elevated levels of Ca^{2+}-bound CaM are associated with cancer [Hait and Lazo, 1986]. In contrast, CaM antagonists inhibit tumor cell invasion in vitro and metastasis in vivo [Ito et al., 1991], suggesting that CaM antagonists are promising chemotherapeutic agents for malignancies. Consistent with these observations, we found that CaM antagonists promote apoptosis of cholangiocarcinoma cells [Pan et al., 1999]. Further, we have shown that CaM antagonists induce apoptosis of cholangiocarcinoma cells, at least in part, through Fas-mediated apoptosis pathways [Ahn et al., 2003]. Recently, we have identified a Ca^{2+}-dependent interaction between CaM and Fas in Jurkat cells and osteoclasts that is regulated during Fas or CaM antagonists-induced apoptosis [Ahn et al., 2004; Wu et al., 2005]. In combination, these observations support the hypothesis that CaM is involved in regulation of Fas-mediated apoptosis through direct interaction with Fas. Thus, in the present studies we investigated whether CaM interacted with Fas in cholangiocarcinoma cells and its potential role in regulating Fas-mediated apoptosis.

The molecular mechanisms of the apoptosis pathways mediated by the prototypic death receptor, Fas, are well studied [Nagata, 1997]. Fas death receptor is a member of the tumor necrosis factor (TNF) receptor superfamily. Upon stimulation by its ligand (FasL) or agonistic antibody, Fas induces receptor clustering and formation of a DISC. This complex recruits, the adaptor molecule, Fas-associated death domain protein (FADD) and procaspase-8 molecules [Muzio et al., 1996; Algeciras-Schimnich et al., 2002]. Caspase-8 is the main initiator caspase in Fas-mediated apoptosis signaling. Once recruited into the DISC, high local concentrations of procaspase-8 lead to its autoproteolytic cleavage and activation, which in turn activates downstream signals [Kischkel et al., 1995; Salvesen and Dixit, 1999]. Depending on the quantity of caspase-8 at the DISC, two types of Fas-mediated apoptosis pathways have been identified [Scaffidi et al., 1998]. In Type I cells, a high concentration of caspase-8 at the DISC can activate the effector caspases, such as caspase-3 and -7. In contrast, in Type II cells, only small amount of caspase-8 is present in the DISC and the caspase-8 signaling is enhanced via the mitochondrial amplification mechanisms, which leads to the cleavage of the BH-3-only protein Bid, release of cytosolic cytochrome *c* and activation of caspase-9 [Scaffidi et al., 1998]. Activation of these downstream caspases directs the apoptotic program into a multitude of subprograms to cleave specific death substrates, thus inducing apoptosis. Accordingly, DISC formation and subsequent protein recruitment is the most critical initial step in regulating Fas-mediated apoptosis. Based on the observation that CaM interacts directly with Fas and this interaction is regulated during Fas-mediated apoptosis in Jurkat cells [Ahn et al., 2004], we hypothesized that CaM may affect Fas-mediated apoptosis by regulating the formation of DISC. To test this hypothesis, the recruitment of CaM to the DISC in cholangiocarcinoma cells in response to Fas stimulation was analyzed.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents

The cholangiocarcinoma cell line, Sk-ChA-1, was kindly provided by Dr. A. Knuth (Ludwig Institute for Cancer Research, London, UK). Cells were grown in RPMI 1640 (Invitrogen) supplemented with penicillin (50 U/ml), streptomycin (50 μg/ml), and 10% heat-inactivated fetal bovine serum. Experiments were performed in Dulbecco's Modified Eagle Medium (DMEM) with Hams' nutrient mixture F12 (DMEM/F12, Gibco-BRL).

Fas agonistic antibody (CH-11, mouse IgM, Upstate Biotechnology, Lake Placid, NY) at 500 ng/ml was used to activate the Fas-mediated signaling pathway. Purified CaM protein (Calbiochem) was used to determine the direct interaction of CaM and Fas. Latrunculin A (Calbiochem) was used to interrupt actin formation. The CaM antagonist, TFP, was purchased from Sigma (St. Louis, MO).

Determination of Intracellular Calcium Change Upon Fas Activation

The effect of Fas activation on intracellular calcium was determined by immunofluorescent staining using FLUO4-AM (Invitrogen) and analyzed with fluorescent microscopy according to the manufacture's recommendation. Briefly, cholangiocarcinoma cells seeded in a 35 mm glass bottom dish with a 14 mm well inside were

washed with phosphate-buffered saline (PBS). Cells were then incubated with FLUO4-AM in Hanks Balanced Salt Solution with dextrose (HBSSD) in dark for 60 min at room temperature and then cell images were analyzed under fluorescent microscope to determine the baseline calcium concentration. Subsequentially cells were exposed to CH-11 (500 ng/ml) and images were taken of the same field for up to 60 min. Mouse IgM was used as control. The mean density of the images was analyzed for relative intracellular calcium concentration.

Characterization of the Interaction Between CaM and Fas

The interaction between CaM and Fas was characterized as we previously reported by: (A) Protein pull-down assay [Ahn et al., 2004]; (B) Immunoprecipitation [Ahn et al., 2004]; (C) Immunostaining and confocal microscopy [Chen et al., 2004]; and (D) ELISA-based quantitative analysis [Chen et al., 2004].

Protein pull-down assay. Cells were washed twice with cold PBS and lysed on ice in lysis buffer containing 100 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% sodium dodecyl sulfate (SDS), 10% glycerol, 1% triton X, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM β-glycerophosphate and protease inhibitor cocktail tablets (Roche, Indianapolis, IN). After determination of the concentrations of protein with the Bicinchoninic acid kit (Sigma), 100 μg of extracted protein from cholangiocarcinoma cells were incubated with 30 μl of CaM-Sepharose or control-Sepharose (Sigma) beads overnight at 4°C. The beads were then washed 4–5 times with lysis buffer and boiled in (30 μl) 2× Laemmli sample buffer. The eluted proteins were subjected to a 10% SDS-PAGE for Western blotting analysis of CaM and Fas.

Immunoprecipitation. Two-hundred micrograms of extracted proteins were incubated with 1 μg of anti-CaM antibody [Sacks et al., 1991] (Upstate Biotechnology, Lake Placid, NY). Immune complexes were recovered from the supernatant by incubation with 30 μl of 1:1 slurry of protein A-Sepharose beads (Pierce Biotechnology, Inc., Rockfort, IL) overnight at 4°C. Beads were then washed with lysis buffer, and 20 μl 2× Laemmli sample buffer was added to the beads followed by heating at 95°C for 5 min and chilling on ice. After brief centrifugation, proteins in the supernatant were analyzed for CaM and Fas by Western blot with specific antibodies.

Immunolocalization of CaM and Fas with confocal microscopy. To determine the colocolization of CaM and Fas in response to Fas stimulation, immunostaining and confocal microscope analyses were performed as we previously described [Chen et al., 2004]. Cholangiocarcinoma cells were incubated with or without CH-11 for 30 min and subsequentially fixed in formaldehyde and stained by anti-CaM and anti-Fas antibody (B-10). The stained cells were examined by confocal microscopy. Images were captured in sequential mode in the following manner. 492-nm was used to excite the Cy2 fluorescence (indicating staining with the anti-CaM antibody) and the 594-nm was used to excite the Alexa Fluor 594 fluorescence (Molecular Probe, indicating staining for anti-Fas antibody). The two images were electronically merged to produce a pseudo colored image in which green depicts CaM, red depicts Fas immunoreactivity and yellow depicts co-localization of CaM and Fas.

ELISA-based quantitative analysis of CaM and Fas binding. A quantitative method as we previously published [Chen et al., 2004], was also employed to determine the interaction between Fas and CaM. Briefly, 50nM purified GST-Fas protein [Ahn et al., 2004] was diluted in HEPES buffered saline (HBS, 20 mM HEPES, 150 mM NaCl, pH 7.5) with 5 μg/ml BSA and applied to 96 well plates to incubate overnight for passive adsorption of proteins to the wells. The plates were washed and blocked with HBS plus 2% BSA. Equivalent coating efficiency under these conditions was assayed by ELISA with the use of an anti-GST antibody. To assess CaM binding, purified CaM at the concentrations of 0–50 nM was applied to GST-Fas-coated wells. The plate was incubated with anti-CaM monoclonal antibody and then HRP-conjugated anti-mouse IgG (Sigma) to detect CaM bound to Fas. GST only-coated wells served as negative controls.

Analysis of DISC

Immunoprecipitation for DISC analysis was performed by a modification of a previously published protocol [Gomez-Angelats and Cidlowski, 2001; Scaffidi et al., 2000]. Cells, 5×10^7, were incubated with 1 μg/ml Fas activating antibody (CH-11) for 30 min at 37°C and then washed with PBS and lysed in lysis buffer for 30 min on ice. In

control cells, Fas activating antibody (CH-11) was added to cell lysates at a final concentration of 1 μg/ml to immunoprecipitate non-stimulated Fas receptors. After centrifugation at 15,000*g* for 15 min at 4°C, the supernatant was immunoprecipitated with 20 μl of goat anti-mouse IgM-agarose (Sigma) overnight at 4°C and analyzed by Western blotting.

Western Blot Analysis

Proteins were separated by a SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon P membranes (Millipore, Bedford, MA). Membranes were blocked in Tris-buffered saline (TBS) containing 5% nonfat milk and 0.1% Tween 20 and incubated with primary antibodies. Anti-GAPDH monoclonal antibody (Research Diagnostics, Inc.) was used to confirm equal loading. Anti-CaM antibody was obtained from Upstate Biotechnology (Lake Placid, NY). All other antibodies including anti-FADD, Fas and caspase-8 were purchased from Cell Signaling Technology (Beverly, MA). The primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech, Piscataway, NJ) and enhanced chemiluminescence detection reagents (Amersham Biosciences, Piscataway, NJ). The density of bands was analyzed with the use of densitometry and Kodak software (Eastman Kodak).

Statistical Analysis

Results are expressed as means ± SD. Differences between two groups were identified with Student's *t*-tests. For multiple groups, one-way analysis of variance and Student–Newman–Keuls tests were used to identify differences. Significance was defined as $P < 0.05$.

RESULTS

Interaction Between Fas and CaM in Cholangiocarcinoma Cells

The interaction between CaM and Fas was identified by protein pull-down assay with the use of CaM-Sepharose beads (Fig. 1A). Multiple forms of Fas were identified in cholangiocarcinoma cells, including Fas at the apparent molecular masses of ~37, ~50, and ~100 kDa. The form of Fas that bound to CaM-Sepharose beads under unstimulated conditions was at apparent molecular masses of ~50 kDa on SDS-PAGE. In contrast, Fas did not bind to control Sepharose beads, indicating a specific binding between CaM and Fas.

The binding of CaM to Fas in cholangiocarcinoma cells was confirmed by immuoprecipitation with the use of anti-CaM antibody (Fig. 1B). To elucidate how the interaction was regulated upon Fas stimulation, cells were exposed to Fas-activating antibody (CH-11) for 0, 15, and 30 min. Immunoprecipitation analyses revealed increased amounts of Fas immunoprecipitated by CaM antibody in cells exposed to CH-11 for 15 and 30 min, suggesting a functional role for such binding in response to Fas stimulation.

The observation was further confirmed with immunostaining and confocal microscope analysis. Under control condition, CaM and Fas distribute evenly in cholangiocarcinoma cells (Fig. 1C). Upon Fas-stimulation, increased membrane localization of both Fas and CaM was demonstrated. Increased co-localization of Fas and CaM to the membrane is consistent with the observation that increase CaM and Fas binding in cells exposed to CH-11 (Fig. 1B).

Affinity of CaM and Fas Binding

The affinity and specificity of CaM binding to Fas was further characterized with the use of a quantitative solid-phase binding assay [Chen et al., 2004]. Purified recombination CaM and GST-Fas proteins [Ahn et al., 2004] were used for the determination, while purified GST were used as negative controls. A concentration-dependent and saturable binding of CaM to Fas was demonstrated (Fig. 2). With the use of GraphPad Prism 4 software, the apparent Kd for CaM binding to Fas was estimated to be ~8 nM (Fig. 2, inset).

CaM Binds to Fas Microaggregates Upon Fas Stimulation

It is well established that Fas oligomerizes upon activation to form SDS-stable microaggregates on SDS-PAGE [Algeciras-Schimnich et al., 2002]. We have shown that the major form of Fas that interacts with CaM are at an apparent mass of ~50 kDa (Fig. 1A and B). To determine whether CaM also bound to microaggregates of Fas, immunoprecipitation was performed with the use of anti-CaM antibodies after Fas-stimulation. Consistent with our observation in Figure 1B, increased binding of

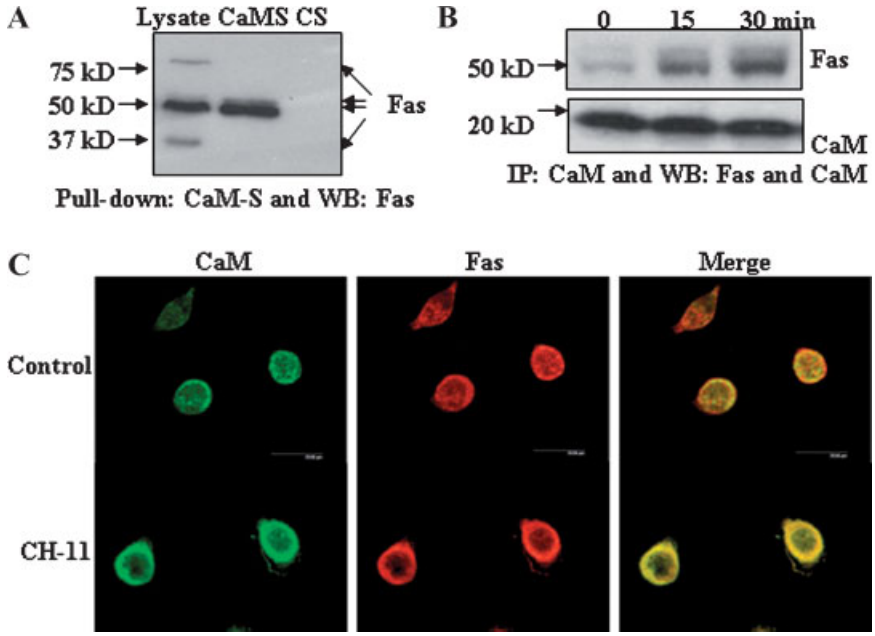

Fig. 1. Interaction between CaM and Fas in cholangiocarcinoma cells. **A**: Protein pull-down assay. Cholangiocarcinoma cells were incubated in growth media to 80% confluence. Cells were lysed. The **left lane** shows the expression of Fas (by Western blot) in the cell lysates. In the **middle** and **right lanes**, cell lysates were incubated with CaM-bound Sepharose beads (CaMS) or control Sepharose beads (CS). In the middle lanes, the forms of Fas pulled-down by CaM are demonstrated. Representative blots of six independent determinations are shown. **B**: Immunoprecipitation. Cholangiocarcinoma cells were exposed CH-11 for 0, 15,and 30 min and immunoprecipitation was performed with anti-CaM antibody. The amount of CaM pulled-down by anti-CaM antibody in cells exposed to CH-11 for 0, 15,and 30 is shown in the **lower panel** and Western blot analysis of the Fas that bound to CaM in response to Fas activation is demonstrated in the **upper panel**. Representative blots of three experiments are shown. **C**: Immunolocalization. After incubation with or without Fas agonist antibody (CH-11) for 30 min, cells were fixed in formaldehyde and stained by anti-CaM and anti-Fas antibody (B-10). The stained cells were examined by confocal microscopy. Images were captured in sequential mode in the following manner. The 492-nm was used to excite the Cy2 fluorescence (indicating staining with the anti-CaM antibody) and the 594-nm was used to excite the Alexa Fluor 594 fluorescence (indicating staining for anti-Fas antibody). The two images were electronically merged to produce a pseudocolored image in which green depicts CaM, red depicts Fas immunoreactivity and yellow depicts co-localization of CaM and Fas. Representative images of three independent experiments are shown.

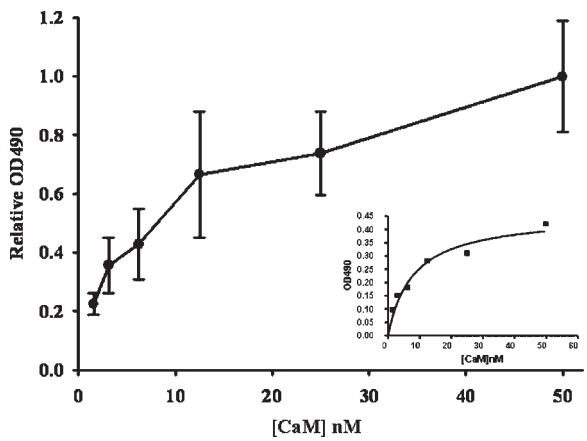

Fig. 2. Determination of the interaction between CaM and Fas with purified recombinant proteins. The relative binding affinity of CaM and Fas was evaluated by a quantitative colorimetric binding assay. The absorbance at 490 nm for CaM and GST-Fas coated wells determined for each CaM concentration were corrected for nonspecific binding of CaM to GST only-coated wells and then normalized with respect to 50 nM CaM value (defined as 1). Results were analyzed with the use of GraphPad Prism 4 software to estimate an apparent Kd for the interaction between CaM and Fas (inset). Results shown are from three independent experiments performed in duplicate.

CaM to Fas with apparent molecular mass of ~50 kDa was demonstrated after cholangiocarcinoma cells were exposed to CH-11 for 15, 30, 60, and 120 min (Fig. 3). More interestingly, increased binding of CaM to Fas microaggregates at apparent masses of 75 to over 100 kDa was demonstrated in cells exposed to CH-11 (Fig. 3). Within the 120 min examined, the size of Fas microaggregates bound to CaM was increased in a time-dependent manner after Fas activation (Fig. 3).

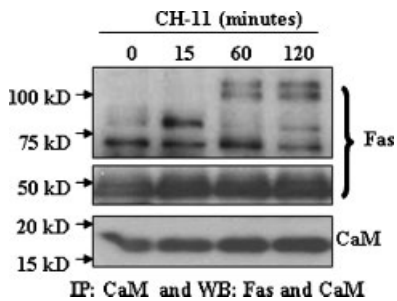

Fig. 3. Interaction of CaM with Fas microaggregates in response to Fas stimulation. Cholangiocarcinoma cells were exposed to Fas agonist antibody CH-11 for 0, 15, 60, and 120 min. Immunoprecipitation was performed in extracted proteins with the use of CaM antibody and Western blot analyses were used to identify the forms of Fas that interacted with CaM after Fas-stimulation (identified as } Fas. The blot was split into upper and lower parts and developed for different lengths of time to achieve better exposure for each part). Representative blot showing the expression of CaM is used as control for Immunoprecipitation (n = 3).

Fas-Mediated DISC Analysis in Cholangiocarcinoma Cells

The death cascade mediated by Fas involves the formation of the DISC upon Fas stimulation, which recruits death effectors and activates downstream catabolic pathways [Nagata, 1997]. We analyzed the Fas-induced DISC and found the recruitment of the adaptor protein FADD and death effector caspase-8 into the DISC in cholangiocarcinoma cells exposed to Fas-activating antibody CH-11 for 15 min (Fig. 4A). Increased FADD and caspase-8 was recruited into the DISC after the cells were exposed to CH-11 for 15, 30, and 60 min.

Since we observed an increased binding of CaM and Fas within the time frame of DISC formation after Fas stimulation (Figs. 1B and 4A), we determined whether CaM was recruited into the DISC. By analyzing proteins recruited into the DISC, we were able to detect the presence of CaM in DISC (Fig. 4B). More importantly, the presence of CaM in the DISC

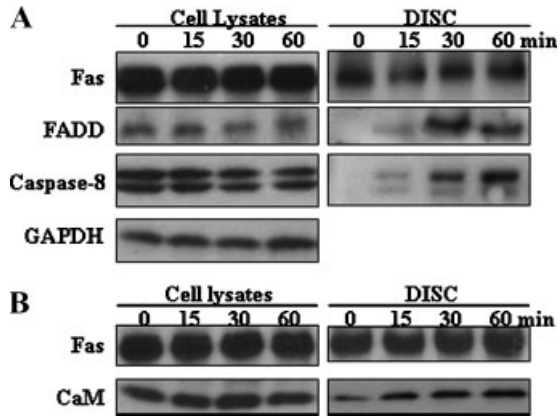

Fig. 4. DISC analysis of cholangiocarcinoma cells exposed to Fas activating antibody CH-11. **A**: Cholangiocarcinoma cells were grown to 80% confluence and exposed to CH-11 for 0, 15, 30, and 60 min. Western blot analyses were performed with extracted proteins to determine the expression of DISC proteins including Fas, FADD, and caspase-8 in response to CH-11 (Cell lysates, n = 6). The expression of GAPDH was used as loading control. Immunoprecipitation of DISC complex were performed to determine the recruitment of Fas, FADD and caspase-8 into DISC in response to CH-11 (DISC, n = 6). **B**: The expression (cell lysate) and DISC recruitment (DISC) of CaM was determined in cholangiocarcinoma cells exposed to CH-11 for 0, 15, 30, and 60 min. Representative blots of four independent experiments are shown.

was increased after cells were exposed to CH-11 for 15, 30, and 60 min (Fig. 4B), which is correlated with the increase in recruitment of other DISC proteins, FADD and caspase-8 (Fig. 4A).

Determination of Intracellular Calcium Upon Fas Stimulation

We have previously reported that the CaM binds to Fas in a calcium-dependent manner [Ahn et al., 2004]. Increased intracellular calcium was also demonstrated in cholangiocarcinoma cells after exposure to CH-11 for a period of 4 and 8 h [Ahn et al., 2002]. To elucidate whether increased intracellular calcium contributes to the increased recruitment of CaM into Fas-mediated DISC upon Fas stimulation, we determined intracellular calcium after cholangiocarcinoma cells were exposed to CH-11 for 0–60 min. Elevation of intracellular calcium was demonstrated in cholangiocarcinoma cells in response to Fas-stimulation in a time-dependent manner (Fig. 5). The intracellular calcium was not affected by addition of mouse IgM (data not shown). The intracellular calcium level increased rapidly within 5 min after CH-11 stimulation and gradually decreased after 30 min.

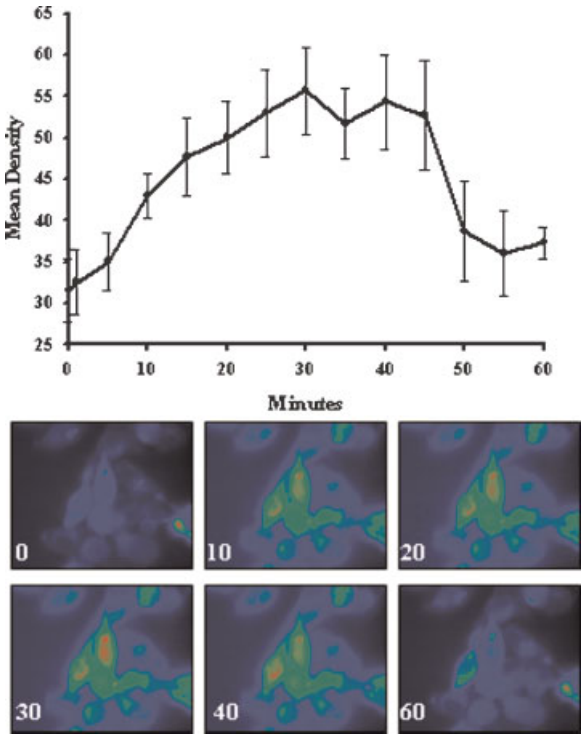

Fig. 5. Determination of intracellular calcium in cholangiocarcinoma cells in response to CH-11 stimulation. Cholangiocarcinoma cells grown in a 35 mm glass bottom dish with a 14 mm well inside were washed with PBS and incubated with FLUO 4 in the dark for 60 min. Cell images were taken with a fluorescent microscope to determine the baseline calcium concentration (time 0, defined as 1). Subsequentially cells were exposed to CH-11 (500 ng/ml) and images were taken of the same field for up to 60 min. The relative mean densities of the images at each time point is shown (n = 3). Representative images of three independent experiments are shown. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Effects of EGTA and TFP on the DISC Recruitment of CaM

Previous studies from our group have demonstrated that the interaction of CaM and Fas is Ca^{2+}-dependent [Ahn et al., 2004]. Thus, we further verified whether Ca^{2+} affected the recruitment of CaM into Fas-mediated DISC. DISC analyses were performed with or without calcium chelator EGTA. The recruitment of CaM to the Fas-mediated DISC was inhibited in the presence of EGTA (Fig. 6A), confirming that the recruitment of CaM into the DISC is calcium-dependent. In addition, we examined the effect of the CaM antagonist TFP on the recruitment of CaM into the DISC (Fig. 6B). In the absence of TFP, increased CaM was present in the DISC after cells were exposed to CH-11 for up to 60 min (Fig. 6B, left 3 lanes). By

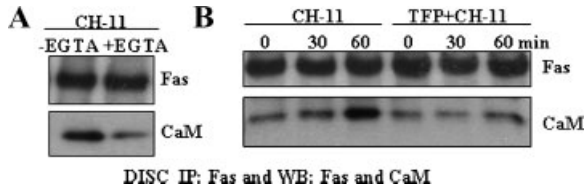

Fig. 6. Effects of EGTA and TFP on the DISC recruitment of CaM. **A**: Effect of EGTA on the recruitment of CaM and into DISC. Cholangiocarcinoma cells were preincubated with or without EGTA (2 mM) for 30 min and then exposed to CH-11 for 30 min. Western blot analyses were performed to determine the DISC recruitment of CaM. Representative blots of three independent experiments are shown. **B**: Effect of TFP on the recruitment of CaM into the DISC. Cholangiocarcinoma cells were preincubated with or without TFP (10 μM) for 30 min and subsequentially exposed to CH-11 for 0, 30, and 60 min. DISC analyses were performed to determine the effect of TFP on recruitment of CaM into the Fas-mediated DISC. Representative blots of three independent experiments are shown.

contrast, pre-incubation of cells with TFP before CH-11 treatment inhibited the increase of CaM in the DISC (Fig. 6B, right 3 lanes).

Effect of Inhibition of Actin Formation on the Recruitment of CaM to the DISC

Previous studies from Peter and colleagues suggest that actin mediates the recruitment of the DISC [Algeciras-Schimnich et al., 2002]. Accordingly, we determined the effects of Latrunculin A, a pharmacological inhibitor of actin formation, on the expression of Fas and CaM as well as on the recruitment of CaM into the DISC upon Fas stimulation in cholangiocarcinoma cells. We found that Latrunculin A did not affect the expression of Fas or CaM in cholangiocarcinoma cells (Fig. 7A). However, the recruitment of CaM into the DISC was abolished when cells were pre-incubated with Latrunculin A for 30 min (Fig. 7B). Latrunculin A inhibited the CaM recruitment to the DISC under both unstimulated and CH-11-stimulated conditions.

Effects of TFP on Cleavage of Caspase-8

DISC recruitment of caspase-8 (p55/53) leads to its autoproteolytic cleavage and activation [Salvesen and Dixit, 1999]. As shown above in Figure 3, increased Fas-recruitment of caspase-8 was demonstrated after Fas stimulation. Accordingly, we determined the DISC-induced cleavage of caspase-8, as well as the effect of TFP on the cleavage of caspase-8. CH-11 induced the cleavage of caspase-8 in a time-dependent manner, as evident by the increased cleavage of caspase-8 after 30, 60, and 120 min

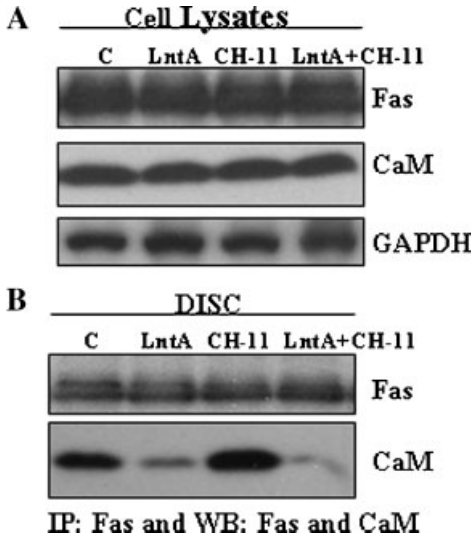

Fig. 7. Inhibition of actin formation abolished recruitment of CaM into the DISC. **A**: Effect of actin formation inhibition on the expression of Fas and CaM in cholangiocarcinoma cells. Cholangiocarcinoma cells were preincubated with or with out 5 μm Latrunculin A for 30 min and then exposed to CH-11 for 30 min. Western blot analyses were performed to determine the expression of Fas and CaM (n = 3). **B**: Effect of actin formation inhibition on the recruitment of CaM into the Fas-mediated DISC. The recruitment of CaM into DISC was analyzed in the DISC complex from cholangiocarcinoma cells exposed to CH-11 with or without preincubation with Latrunculin A. Representative blots of three independent determinations are shown.

(Fig. 8A). However, when cells were exposed to CH-11 in the presence of TFP for 30, 60, and 120 min, the CH-11-induced cleavage of caspase-8 was inhibited (Fig. 8B).

DISCUSSION

Studies from our group have supported a role of CaM in Fas-mediated apoptosis in several cell types, including cholangiocarcinoma [Pan et al., 1999; Ahn et al., 2003, 2004; Wu et al., 2005]. Recently, we have identified direct binding between Fas and CaM in Jurkat cells and osteoclasts, and demonstrated that the CaM and Fas binding is regulated during Fas and CaM antagonist-induced apoptosis [Ahn et al., 2004; Wu et al., 2005]. In response to Fas stimulation, CaM and Fas binding is increased within 30 min and then decreased after 2 h in Jurkat cells [Ahn et al., 2004]. The CaM and Fas binding in osteoclasts is inhibited after cells were exposed to the CaM antagonist, TFP, for 15 min and gradually recovered at 60 min [Wu et al., 2005]. However, it is not clear how the regulation of CaM and Fas binding by Fas stimulation and/or CaM antagonists affects Fas-mediated apoptosis pathways. In the present studies, we determined the interaction between CaM and Fas that is increased in response to Fas stimulation in cholangiocarcinoma cells. The binding of CaM and Fas in three different cell types suggests a fundamental role of such interaction in regulating cell function. Accordingly, we began elucidating the mechanism responsible for the regulation of CaM and Fas binding in Fas-mediated apoptosis signaling pathways.

The immunohistological identification of membrane co-localization of CaM and Fas upon Fas stimulation confirmed the hypothesis that CaM and Fas binding is involved in Fas-mediated DISC formation. Fas oligomerization, as evident by increased membrane localization, is an important initial step upon triggering by Fas ligand (FasL) or Fas agonist antibody [Algeciras-Schimnich et al., 2002]. CaM is a small intracellular protein that mainly functions as an intracellular mediator of the Ca^{2+} signals induced by extracellular stimuli [Moser et al., 1995]. The membrane or peri-membrane

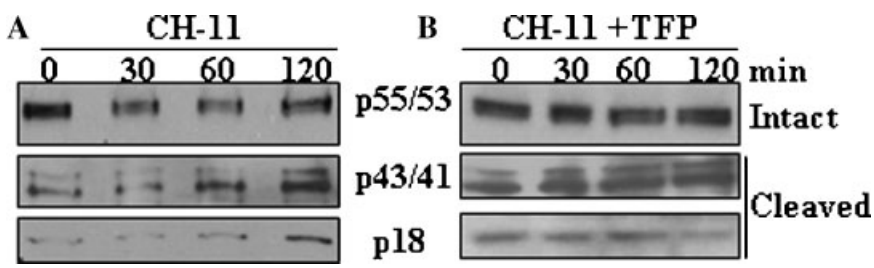

Fig. 8. Effects of TFP on the cleavage of caspase-8. **A**: Cleavage of caspase-8 upon Fas stimulation. Cholangiocarcinoma cells were exposed to CH-11 for 0, 30, 60, and 120 min. Western blot analysis was performed using anti-caspase-8 antibody. Representative blots of three independent experiments are shown. **B**: Effect of TFP on the cleavage of caspase-8. Cholangiocarcinoma cells were exposed to CH-11 with TFP (10 μM) for 0, 30, 60, and 120 min. Western blot analysis of caspase-8 was performed. Representative blot of three independent experiments is shown.

localization of CaM in cholangiocarcinoma cells upon Fas stimulation suggested that CaM might be involved in Fas oligomerization or related molecular events in mediating Fas-stimulated apoptosis signaling. CaM bound to Fas prior to Fas oligomerization, as demonstrated by the binding of CaM to Fas at apparent molecular masses of ~50 kDa under unstimulated condition (Fig. 1) [Ahn et al., 2004; Wu et al., 2005]. Consistent with previous observations by Peter and colleagues that Fas oligomerization happens 15 min after stimulation [Algeciras-Schimnich et al., 2002], we found that CH-11-induced Fas microaggregates, with increased apparent molecular mass, bound to CaM 15–30 min after Fas stimulation (Fig. 3).

During Fas-mediated apoptosis, oligomerized Fas recruits the death receptor FADD, which in turn recruits caspase-8 [Nagata, 1997]. Similarly, we found increased recruitment of FADD and caspase-8 into Fas-mediated DISC in cholangiocarcinoma cells (Fig. 4A). More importantly, we identified the recruitment of CaM into Fas-mediated DISC, which further supported our hypothesis that CaM and Fas binding may be involved in DISC formation. Crystal and solution structures have indicated that CaM is composed of two homologous globular domains connected by a long central alpha-helix that is flexible in solution. Each globular domain consists of two EF-hand Ca^{2+}-binding sites that interact via a short beta-sheet [Babu et al., 1985]. Ca^{2+}-bound CaM activates target enzymes, such as Ca^{2+}/CaM-dependent kinase II [Colbran et al., 1988] and calcineurin [Klee et al., 1979], which play important roles in a variety of cell functions. Fas stimulation has been shown to increase intracellular Ca^{2+} in a variety of cells, including FMO cells [Oshimi and Miyazaki, 1995], Jurkat cells [Sen et al., 1999], and cholangiocarcinoma cells [Ahn et al., 2003]. In all these studies, a substantial elevation of intracellular Ca^{2+} has been detected between 1 and 4 h after Fas stimulation [Ahn et al., 2003; Oshimi and Miyazaki, 1995; Sen et al., 1999]. In addition, Oshimi et al. has observed an early response of intracellular Ca^{2+} at 9 ± 4 min that lasted for 15–20 min after Fas stimulation [Oshimi and Miyazaki, 1995]. This is similar to what we found in cholangiocarcinoma cells, that intracellular Ca^{2+} increased within 5 min after CH-11 stimulation and gradually decreased after 30 min to baseline by 60 min (Fig. 5). Because CaM binding to Fas is Ca^{2+}-dependent [Ahn et al., 2004; Wu et al., 2005], increased intracellular Ca^{2+} immediately after Fas stimulation likely contributes to the increased presence of CaM in Fas-mediated DISC; whereas EGTA decreased the recruitment of CaM into Fas-mediated DISC (Fig. 5). Furthermore, we found that the CaM antagonist, TFP, blocked, or at least delayed, the recruitment of CaM into Fas-mediated DISC (Fig. 6). This observation is consistent with our previous determination in osteoclasts that TFP decreases the CaM and Fas binding [Wu et al., 2005].

In addition, we found that Latrunculin A inhibited the recruitment of CaM into the Fas-mediated DISC (Fig. 7). Latrunculin A is a natural compound purified from *Latrunculia manificans*. It has been shown to alter the actin-monomer subunit interface thus preventing actin polymerization [Morton et al., 2000]. Inhibition of actin cytoskeleton by Latrunculin A disrupts the Fas-mediated DISC and receptor internalization [Algeciras-Schimnich et al., 2002]. This likely explains why the recruitment of CaM into Fas-mediated DISC was decreased in the presence of Latrunculin A. However, we also observed a decrease in CaM and Fas binding by Latrunculin A in unstimulated conditions. Actin-binding protein filamin A is the most potent actin filament (F-actin) cross-linking protein known to promote perpendicular branching of actin filaments into three-dimensional gelled network [Nunnally et al., 1981]. Recently, a direct interaction of CaM and filamin A was demonstrated [Nakamura et al., 2005]. Accordingly, when the actin skeleton was dissociated by Latrunculin A, increased free filamin A might bind to CaM causing a decrease in CaM and Fas binding.

CaM has been demonstrated to bind to the Fas DD [Ahn et al., 2004], which is the region that Fas interacts with several other proteins isolated in yeast two-hybrid screens, including FADD, receptor-interacting protein kinase 1 (RIP 1) [Stanger et al., 1995], Fas-associated phosphatase-1 (FAP-1) [Sato et al., 1995], Fas-associated factor 1 (FAF 1) [Chu et al., 1995] and death-associated protein (DAXX) [Yang et al., 1997]. Among these Fas-binding proteins, FADD is the only one known to be recruited into the DISC, mediating Fas-induced apoptosis signaling. The role of the interaction of other proteins with Fas in regulating DISC and/or Fas-mediated apoptosis signaling pathways has

not been defined, although they have been shown to negatively or positively affect Fas-mediated apoptosis [Chu et al., 1995; Stanger et al., 1995; Yang et al., 1997; Sato et al., 1995]. The data presented in our studies suggests that CaM and Fas binding appear to be involved in Fas-induced DISC formation, thus regulating Fas-mediated apoptosis pathways. First, Fas oligomerization is the molecular event triggering DISC formation in response to Fas stimulation and Fas micro aggregates bind to CaM upon Fas stimulation. Second, DISC analyses revealed increased CaM after Fas-stimulation, which was inhibited when the DISC was disrupted. Further, CaM and Fas binding is Ca^{2+}-dependent. Increased intracellular calcium induced by Fas was associated with the increased presence of CaM in DISC, whereas EGTA inhibited CaM in the DISC. Another well established DISC component is caspase-8, the main initiator caspase in Fas-mediated apoptosis signaling pathways [Peter and Krammer, 2003]. Recruitment of caspase-8 into the DISC induces autoproteolytic cleavage of caspase-8 and its activation [Salvesen and Dixit, 1999]. In cholangiocarcinoma cells, increased cleavage of caspase-8 was identified immediately after Fas stimulation (Fig. 8A). TFP, however, inhibited Fas-induced cleavage of caspase-8 (Fig. 8B), which was associated with the inhibition of the recruitment of CaM into the Fas-mediated DISC (Fig. 5). Apparently, the inhibition of Fas-stimulated increased CaM and Fas binding by TFP was associated with inhibition of DISC-induced cleavage of caspase-8.

The molecular mechanism responsible for CaM antagonists on cell apoptosis has not yet been fully elucidated. CaM antagonists have different effects on cell apoptosis in various cell types. We have previously reported that CaM antagonists, TFP and tamoxifen, protect against apoptosis in CD4+ T cells from AIDS patients [Pan et al., 1998]. On the other hand, we and others has demonstrated that CaM antagonists promote apoptosis in many other cell lines [Kang et al., 1999; Pan et al., 1999; Ahn et al., 2003]. Clearly, the expression and activation of other molecules downstream of CaM activation play important roles in determining cell responses to apoptotic stimuli. Several Ca^{2+}/CaM activated enzymes, such as Ca^{2+}/CaM-dependent kinase II (CaMKII)[Colbran et al., 1988; Yang et al., 2003], calcineurin [Klee et al., 1979; Michalak et al., 1999] and the death-associated protein kinase [Deiss et al., 1995] play important roles in a variety of cell functions, including apoptosis. Recently, studies from Hao and colleagues have demonstrated that CaMKII upregulates the expression of FLIP, which mediates the resistance of astrocytes and glioma cells to Fas-mediated apoptosis [Yang et al., 2003; Song et al., 2006]. Consistent with this, we found that the expression of FLIP is critical for determining life or death of cells in response to TNF/Fas signaling in cholangiocarcinoma cells [Chen et al., 2006b] and in vascular smooth muscle cells [Chen et al., 2006a]. Micheau et al. [2001] has reported that the expression of caspase-8 is quite stable and modest changes in expression of FLIP appear to determine whether a cell proliferates or dies in response to selected stimuli. Accordingly, CaM antagonists may regulate apoptosis by affecting the expression of FLIP. In addition, we have demonstrated that CaM antagonists also induce apoptosis of cholangiocarcinoma cells through a caspase-independent pathway by inducing depolarization of the mitochondrial membrane [Ahn et al., 2003]. Thus, inhibition of recruitment of CaM into Fas-activated DISC and subsequent caspase-8 activation by TFP is consistent with our previous observations that TFP induce apoptosis of cholangiocarcinoma cells through additional mechanisms independent of caspase-8 activation.

In summary, we demonstrated that the Ca^{2+}-dependent recruitment of CaM into the DISC was increased in cholangiocarcinoma cells upon Fas-stimulation. Calcium chelator, EGTA, and CaM antagonist, TFP, decreased Fas-induced DISC recruitment of CaM and DISC-induced cleavage of caspase-8 was inhibited by TFP. These results suggest an important role of CaM in mediating Fas-induced DISC formation and apoptotic signaling in cholangiocarcinoma cells. Further characterization of the molecular mechanism of the role of CaM in regulating Fas-mediated apoptosis could have implications for novel therapies for cholangiocarcinoma.

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