CpG-B Oligodeoxynucleotide Promotes Cell Survival via Up-regulation of Hsp70 to Increase Bcl-xL and to Decrease Apoptosis-inducing Factor Translocation

Received for publication, June 7, 2006, and in revised form, September 26, 2006 Published, JBC Papers in Press, October 17, 2006, DOI 10.1074/jbc.M605439200

Cheng-Chin Kuo, Shu-Mei Liang, and Chi-Ming Liang

From the Agricultural Biotechnology Research Center, the Genomics Research Center, and the Institute of Biological Chemistry, Academia Sinica, Taipei 115, Taiwan and the Graduate Institute of Biotechnology, National Chung-Hsing University, Taichung City 402, Taiwan

Unmethylated CpG oligodeoxynucleotides (ODNs) activate immune responses in a TLR9-dependent manner. In this study, stimulation of mouse macrophages with CpG-B ODN increased cellular Hsp70 expression and prevented apoptosis induced by serum starvation or staurosporine treatment. CpG-B ODN-induced Hsp70 expression depended on TLR9, MyD88, and phosphatidylinositol 3-kinase. Inhibition of Hsp70 synthesis by an inhibitor (quercetin) or antisense hsp70 attenuated not only Hsp70 expression but also the anti-apoptotic capacity of CpG-B ODN. Ectopic expression of Hsp70 rescued the inhibitory effect of quercetin on CpG-B ODN-induced anti-apoptosis. Additional experiments demonstrated that quercetin and antisense hsp70 modulated CpG-B ODN-induced anti-apoptosis via a caspase-3-independent pathway by down-regulating the survival gene bcl-xL and by increasing translocation of apoptosis-inducing factor. These findings suggest that CpG-B ODN may up-regulate Hsp70 via a TLR9/MyD88/phosphatidylinositol 3-kinase pathway to increase Bcl-xL and to decrease apoptosis-inducing factor nuclear translocation, resulting in anti-apoptosis.

Like the unmethylated CpG motif in bacterial and viral DNAs, synthetic CpG oligodeoxynucleotides (ODNs) can bind to TLR9 (Toll-like receptor 9) and activate immune responses (3). Activation of TLR9 requires the acidification of endosomes and lysosomes (4), which subsequently initiates a signaling cascade involving MyD88 (myeloid differentiation factor 88). Recruitment of MyD88 is followed by the engagement of IRAK (interleukin-1 receptor-associated kinase) and its adaptor protein, TRAF6 (tumor necrosis factor receptor-associated factor 6), and activation of transcription factors such as AP1 and NF-κB, resulting in the expression of regulating genes involved in host defense (5, 6).

CpG-B ODNs can be classified into two major classes: CpG-A and CpG-B (1). CpG-A ODNs are effective in activating natural killer cells and stimulating plasmacytoid dendritic cells (pDCs) and macrophages to produce high levels of interferon-α (7, 8). In contrast, CpG-B ODNs primarily stimulate proliferation of B cells as well as secretion of immunoglobulins, interleukin-6, and interleukin-10. CpG-B ODNs also induce maturation and activation of pDCs and macrophages (7, 9). CpG-B ODNs protect B cells and pDCs against spontaneous apoptosis and rescue WHEI-231 B cells from apoptosis induced by IgM (10–12). They also protect mouse spleen cells as well as RAW264.7 macrophages and human RPMI 8226 B cells against γ-irradiation-induced apoptosis (13). However, the molecular mechanisms of the anti-apoptotic effects of CpG-B ODNs remain to be elucidated.

Using proteomic approaches, we found, in a preliminary experiment, that CpG ODNs increase the cellular level of heat shock proteins, notably Hsp70. Heat shock proteins are the most abundant and ubiquitous soluble intracellular proteins and are expressed in both constitutive and inducible forms (14). The primary function of intracellular heat shock proteins appears to be as molecular chaperones to prevent protein aggregation and to contribute to the unfolding of nascent and altered proteins (14). The Hsp70 family contains stress-inducible Hsp70 and constitutively expressed heat shock cognate protein Hsc70. Hsc70 is constitutively abundant in all cells, whereas the level of Hsp70 is low in normal cells. A high level of Hsp70 has been found in cancer or normal cells after stress induction (15, 16). Overexpression of Hsp70 protects cells from apoptotic death induced by not only ischemia, adriamycin, and UVB radiation but also by pathogenic viruses (17–20). Cumulative evidence indicates that cellular damage or stress can induce the expression of Hsp70 to inhibit lysosomal membrane permeabilization (21) and to prevent apoptosis by limiting cellular damage and by accelerating recovery (16, 22). Hsp70 has been reported to exert its anti-apoptotic effects by associating with Apaf1 (apoptotic protease activation factor 1) to prevent...
the formation of a functionally competent apoptosome, thereby inhibiting caspase-9 and caspase-3 activation (23–25). It can also inhibit caspase-independent apoptosis by directly interacting with apoptosis-inducing factor (AIF), thereby preventing AIF-induced chromatin condensation (26, 27). The apoptogenic effect of AIF has been shown to be mediated by a survival gene, bcl-xL (28); however, whether the induced Hsp70 has any effect on Bcl-xL and thereby affects AIF is not well documented.

In this study, we used macrophages and pDCs as models to examine the mechanism of CpG-B ODN-mediated anti-apoptosis. Enhanced expression of Hsp70 but not Hsc70 by CpG-B ODN via the TLR9/MyD88/phosphatidylinositol 3-kinase (PI3K) signaling pathway increased the level of Bcl-xL and decreased AIF nuclear translocation, thereby possibly playing an important role in CpG-B ODN-induced anti-apoptosis.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Phosphorothioate-modified CpG-B ODN and GpC ODN were synthesized by MWG Biotech (Ebersberg, Germany). The ODN sequences that we used on mouse cells were as follows: CpG-B ODN, 5'-TCC ATG ACG TTC CTG ATG CT-3'; and GpC ODN, 5'-TCC ATG AGC TTC CTG ATG CT-3'. The sequence of CpG-B ODN we used on human cells was 5'-TCC ATG ACG TTC CTG ATG CT-3', and that of GpC ODN was 5'-TGG TGC TTT TGT GCT TTT GTG CTT-3'. Quercetin, SB203580, and LY294002 were purchased from Sigma. Anti-Hsp70, anti-Hsc70, anti-Hsp90B, anti-AIF, and anti-Bcl-xL antibodies were purchased from Stressgen Biotechnologies (Victoria, British Columbia, Canada).

**Cell Culture and Cell Treatment**—Human embryonic kidney (HEK) 293T cells and mouse RAW264.7 macrophages were obtained from American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin sulfate, and 200 mmol/liter L-glutamine in a humidified atmosphere of 5% CO₂ at 37 °C. The medium was changed every 2 days for all experiments.

Cells were preincubated with or without inhibitors for 1 h before CpG-B ODN treatment unless specified otherwise. The duration of CpG-B ODN treatment varied depending on the requirement of the experiments.

**pDC and Monocyte/Macrophage Preparation**—pDCs and monocytes/macrophages were prepared from splenocytes of 8-week-old male BALB/c mice. Briefly, spleen cells were depleted of erythrocytes, and pDCs and monocytes/macrophages were then purified with a pDC isolation kit and CD11b MicroBeads (Miltenyi Biotec Inc., Auburn, CA), respectively, according to the manufacturer’s recommendations.

**Plasmid Constructions**—The mouse Tlr9 cDNA was generated by reverse transcription-PCR using total RNA from the mouse RAW264.7 cell line as a template and the following oligonucleotides as primers: 5'-GAG ACT TTC ATG GTT CTC GCT CGA AGG ACT-3' and 5'-CTC GAG CTA TTC TGC GGT AGG TCC-3'. Because the primers incorporate HindIII and Xhol sites, the PCR product was then cloned into HindIII- and Xhol-digested pcDNA3.0 (purchased from Invitrogen) to generate pcDNA3.1-mTLR9.

The myd88 cDNA was generated by reverse transcription-PCR using total RNA from the THP-1 cell line used as a template and the following oligonucleotides as primers: 5'-GGA TCC ATG GCT GCA GGA GGT CCC GCC-3' and 5'-AGG CTT CTC AGG GCA GGG ACA AGG CCT-3'. Because the primers incorporate BamHI and HindIII sites, the PCR product was then cloned into BamHI- and HindIII-digested pcDNA3.1(−) to generate pcDNA3.1(−)-MyD88. To create the dominant-negative (DN) MyD88 construct pcDNA3.1(−)-DN-MyD88, which is a truncated version of MyD88 expressing the N-terminal death domain, pcDNA3.1(−)-MyD88 was used as a template for PCR amplification with a forward primer containing a BamHI restriction site (5'-GGA TCC ATG GCT GCA GGA GGT CCC GCC-3') and a reverse primer containing a HindIII restriction site (5'-AGG CTT AAT GCT GGG CCC CAG CAG). The PC product was cloned into BamHI- and HindIII-digested pcDNA3.1(−).

The mouse hsp70 cDNA was generated by reverse transcription-PCR using total RNA from RAW264.7 cells used as a template and the following oligonucleotides as primers: 5'-GGA TCC ATG GCC AAG ACG GCG ATC-3' and 5'-AGG CTT ATC CAC CTC CTC GAT GGT GGG-3'. Because the primers incorporate BamHI and HindIII sites, the PCR product was cloned into BamHI- and HindIII-digested pcDNA3.1(−) to generate pcDNA3.1(−)-Hsp70. To create pcDNA3.1(−)-Hsp70AS (26), the antisense Hsp70 construct pcDNA3.1(−)-Hsp70 was used as a template for PCR amplification with a forward primer containing a HindIII restriction site (5'--CCC AAG CTT AGG ACG GCG GCG TGA TCG-3') and a reverse primer containing a BamHI restriction site (5'-CCC GGA TCC TTG GCG TCG CGG AGC GCC TCC-3'). The PCR product was cloned into HindIII- and BamHI-digested pcDNA3.1(−). PI3K kinase-deficient plasmid M-p110Δkin-myc was provided by Dr. Anke Klippel (Atugen AG, Berlin, Germany) (29).

**Stable Transfectants**—HEK293T and mouse RAW264.7 cells (5 x 10⁶/well) were transfected with 1 μg of plasmid pcDNA3.1(−)-mTLR9, pcDNA3.1(−)-Hsp70, or pcDNA3.1(−)-Hsp70AS using FuGENE 6 (Roche Applied Science) according to the manufacturer’s instructions. Two days after transfection, the antibiotic G418 (1 mg/ml) was used for clonal selection. The G418-resistant clones were expanded and then screened for expression of mouse TLR9 or Hsp70 by Western blotting.

**Small Interfering RNA Transfection**—Double-stranded RNAs were synthesized by Invitrogen. The target sequence for mouse Hsp90B is 5'-GAG CTT ATG ATA CCT GAG TAC CTC AAC T-3'. They were cloned between the BamHI and HindIII sites downstream of the U6 promoter in the pSilencer2.1-U6 neoplasmin (Ambion, Inc., Austin, TX). The plasmid was transfected into RAW264.7 cells using FuGENE 6. After 48 h, G418 (0.6 mg/ml) was used for clonal selection. The G418-resistant clones were expanded and then screened for expression of mouse Hsp90 by Western blotting.

**Western Blot Analysis of Cell Lysates**—The cells (10⁶/well) were lysed on ice for 15 min with 300 μl of lysis buffer (Pierce) supplemented with protease inhibitor mixture (Sigma). The lysates were centrifuged at 12,000 × g for 15 min at 4 °C, and protein concentrations in the supernatants were determined.
Critical Role of Hsp70 in Anti-apoptosis of CpG-B ODN

using the Bio-Rad protein assay. The supernatants (50–80 μg of protein/lane) were applied to Novex 4–20% Tris/glycine gel (Invitrogen) and transferred to nitrocellulose membranes (Amersham Biosciences) according to the manufacturer’s protocol.

Cell Viability Assay—3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to measure cell viability in terms of metabolic turnover as indicated by the oxidation of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to purple formazan by mitochondria (30). In some cases, trypan blue exclusion assay was used. In brief, 20 μl of cell suspension was mixed with 20 μl trypan blue (Sigma), and cells were then counted using a hemocytometer. The ratio of trypan blue-stained cells to total cells in each experiment was determined by counting four different fields.

Cellular DNA Fragmentation Enzyme-linked Immunosorbent Assay—The level of apoptotic cell death was quantified by cellular DNA fragmentation enzyme-linked immunosorbent assay (Roche Applied Science). The assay was based on the incorporation of the nonradioactive thymidine analog bromodeoxyuridine (BrdUrd) into the genomic DNA. BrdUrd was added to the cell medium at the time of seeding, and the BrdUrd-labeled DNA fragments were released from the cells into the cytoplasm during apoptosis. The DNA fragments were then detected immunologically by enzyme-linked immunosorbent assay using an anti-DNA antibody-coated microplate to capture the DNA fragment and a peroxidase-conjugated anti-BrdUrd antibody to detect the BrdUrd-containing DNA fragments. The degree of apoptosis (cytosolic DNA fragments) was quantified following the manufacturer’s recommendations.

Caspase-3 Activity—Caspase-3 activity was determined by cleavage of the fluorometric substrate benzoyloxycarbonyl-DEVD-aminomethylcoumarin (Upstate, Lake Placid, NY) according to the manufacturer’s instructions. Cells were harvested, washed twice with phosphate-buffered saline, and treated with lysis buffer supplemented with protease inhibitor mixture. The lysates were centrifuged at 12,000 × g for 15 min at 4 °C, and protein concentrations in the supernatants were determined using the Bio-Rad protein assay. The supernatants (50 μl) were assayed in triplicate with 72 μM caspase-3 fluorometric substrate and incubated at room temperature for 15 min. Cleavage of caspase-3 substrate was followed by measurement of emission at 460 nm after excitation at 380 nm using a fluorescence plate reader.

Cell Fractionation—Cytosolic and nuclear fractions for AIF studies were prepared by resuspending cells in ice-cold 250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl2, and protease inhibitor mixture (pH 7.4). Homogenization was performed in a Dounce homogenizer. Nuclei were pelleted at 750 × g for 10 min, and the supernatant was then spun at 10,000 × g for 25 min.

Statistical Analysis—All values are given as the means ± S.D. Data were analyzed by one-way analysis of variance, followed by Scheffe’s test.

RESULTS

CpG-B ODN Up-regulates Cellular Hsp70 but Not Hsc70—Using proteomic approaches, we found, in a preliminary experiment, that CpG-B ODN treatment increased the expression of Hsp70 in swine macrophages. In this study, we further found that the expression of Hsp70 was increased by CpG-B ODN but not GpC ODN (negative control) in the mouse macrophage RAW264.7 cell line and mouse spleen pDCs as well as in monocytes/macrophages (Fig. 1, A and B). The increase in Hsp70 expression occurred mainly intracellularly because the level of Hsp70 in the culture medium showed little, if any, change after CpG-B ODN treatment (data not shown). In addition, we also evaluated the time course of elevation of Hsp70 expression. The level of Hsp70 but not Hsc70 in cells after 14–24 h of CpG-B ODN treatment was increased compared with that in cells treated with GpC ODN (negative control) or medium alone (p < 0.005) (Fig. 1 A).
CpG-B ODN Up-regulates Hsp70 via the TLR9/MyD88/PI3K Pathway—Bacterial CpG DNA has been shown to co-localize in an endosomal compartment with TLR9 after endocytosis (31). To evaluate whether TLR9 and endosomal maturation play a role in CpG-B ODN-mediated induction of Hsp70, we treated a TLR9-deficient HEK293T cell line with CpG-B ODN and found that CpG-B ODN caused little, if any, increase in the cellular Hsp70 level (supplemental Fig. 1). After the mouse Tlr9 gene was stably integrated into HEK293T cells, however, the cellular level of Hsp70 was greatly increased by CpG-B ODN and was modulated by the addition of chloroquine (Fig. 2, A and B), an inhibitor of endosomal maturation known to affect TLR9 function (4).

Because TLR9 signaling is via an MyD88-dependent pathway (32), we further investigated whether the DN-MyD88 mutant would affect the level of Hsp70 induced by CpG-B ODN. RAW264.7 cells were transiently transfected with various concentrations of DN-MyD88 plasmids for 48 h, followed by Western blot analysis of the cell lysates to detect the expression of Hsp70. CpG-B ODN-mediated induction of Hsp70 was inhibited by DN-MyD88 in a dose-dependent manner (Fig. 2 C).

Taken together, these results suggest that CpG-B ODN increases the expression of Hsp70 via a TLR9/MyD88 signaling pathway.

Because CpG DNA/ODN activates PI3K to regulate a variety of cellular responses (29, 33) and because the PI3K/Akt pathway is involved in heat shock protein induction under certain stress conditions (34, 35), we treated RAW264.7 cells with the PI3K inhibitor LY294002 before CpG-B ODN treatment. CpG-B ODN-induced Hsp70 expression was inhibited by LY294002 in a concentration-dependent manner (Fig. 2 D). Consistent with the findings from inhibitor analysis, the CpG ODN-mediated increase in Hsp70 was inhibited by overexpression of a class I PI3K kinase-deficient plasmid (DN-PI3K, i.e. M-p110Δkin-myc), which inhibited the CpG-B ODN-mediated phosphorylation of the PI3K substrate Akt (Fig. 2 E and supplemental Fig. 2). Thus, the PI3K/Akt pathway might be instrumental for CpG-B ODN-mediated induction of Hsp70.

Because CpG DNA/ODN activates PI3K to regulate a variety of cellular responses (29, 33) and because the PI3K/Akt pathway is involved in heat shock protein induction under certain stress conditions (34, 35), we treated RAW264.7 cells with the PI3K inhibitor LY294002 before CpG-B ODN treatment. CpG-B ODN-induced Hsp70 expression was inhibited by LY294002 in a concentration-dependent manner (Fig. 2 D). Consistent with the findings from inhibitor analysis, the CpG ODN-mediated increase in Hsp70 was inhibited by overexpression of a class I PI3K kinase-deficient plasmid (DN-PI3K, i.e. M-p110Δkin-myc), which inhibited the CpG-B ODN-mediated phosphorylation of the PI3K substrate Akt (Fig. 2 E and supplemental Fig. 2). Thus, the PI3K/Akt pathway might be instrumental for CpG-B ODN-mediated induction of Hsp70.

**Induction of Hsp70 via PI3K Is Positively Associated with CpG-B ODN-induced Cell Survival**—Incubation of THP-1 (data not shown) or RAW264.7 (Fig. 3 A) cells in serum-starved medium (0% fetal bovine serum (FBS)) for 60 h resulted in an ~32 or 27% decrease in cell viability, respectively. The addition of CpG-B ODN to cells cultured in serum-starved medium improved the cell viability in a dose-dependent manner, whereas GpC ODN (negative control) had no effect on viability (Fig. 3 A). Incubation of RAW264.7 cells in serum-free medium for >60 h (7 days) resulted in even more cell death (~70%), which was nonetheless still preventable by the addition of CpG-B ODN (Fig. 3 B). This effect of CpG-B ODN was accompanied by a decrease in cellular DNA condensation and fragmentation (supplemental Fig. 3 A), indicating a decline in apoptosis. A similar anti-apoptotic effect of CpG-B ODN was observed in RAW264.7 cells pretreated with the pro-apoptotic agent staurosporine (STS) (supplemental Fig. 3 B) and in pDCs (Fig. 3 C). The anti-apoptotic effect of CpG-B ODN was blocked by the PI3K inhibitor LY294002 but not the p38 MAPK inhibitor SB203580 (Fig. 3 D), suggesting that PI3K plays a more critical role in anti-apoptosis.
Critical Role of Hsp70 in Anti-apoptosis of CpG-B ODN

Inhibition of Hsp70 or PI3K decreases the anti-apoptotic effect of CpG-B ODN. A, RAW264.7 cells were cultured for 60 h in serum (10% FBS) or serum-free medium (0% FBS) with or without various concentrations of GpC ODN or CpG-B ODN as indicated. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. *, p < 0.05 for the increase induced by CpG-B ODN versus GpC ODN (negative control). B, RAW264.7 cells were cultured in serum-free medium (0% FBS) with or without GpC ODN or CpG-B ODN (1 µM) for the indicated times in the presence or absence of 5 µM quercetin (Quer.). C, pDCs were incubated in serum-free medium with or without CpG-B ODN (1 µM) for the indicated times in the presence or absence of 5 µM quercetin. D, RAW264.7 cells were cultured for 60 h in serum (10% FBS) or serum-free medium (0% FBS) with or without GpC ODN or CpG-B ODN (1 µM) in the presence or absence of 20 µM SB203580, 30 µM LY294002, or 5 µM quercetin. E, RAW264.7 cells were cultured for 60 h in serum (10% FBS) or serum-free medium (0% FBS) with or without GpC ODN or CpG-B ODN (1 µM) in the presence or absence of 5 µM quercetin. The extent of apoptotic DNA fragmentation was estimated by measuring the amounts of cytosolic BrdUrd-labeled DNA fragments in enzyme-linked immunosorbent assay. Data represent the means ± S.D. of at least three independent experiments.

To determine whether Hsp70 is indeed involved in CpG-B ODN-mediated anti-apoptosis, we first examined the cellular effects of the Hsp70 synthesis inhibitor quercetin. Although quercetin alone did not affect the viability (Fig. 3B) or DNA fragmentation (Fig. 3E) of RAW264.7 cells, it modulated the anti-apoptotic effect of CpG-B ODN (Fig. 3, B–E and supplemental Fig. 4) and inhibited CpG-B ODN-induced Hsp70 expression (Fig. 4A). Inhibition of Hsp70 expression in transiently transfected RAW264.7 cells with the antisense mouse hsp70 gene also resulted in a decreased CpG-B ODN anti-apoptotic effect (Fig. 4B). Ectopic expression of Hsp70 in RAW264.7 cells decreased the inhibitory effect of quercetin and restored the anti-apoptotic effect of CpG-B ODN (Fig. 4C). Collectively, these results suggest that induction of Hsp70 by CpG-B ODN via the PI3K pathway may play an important role in CpG-B ODN-mediated anti-apoptosis.

Hsp70 Participates in CpG-B ODN-mediated Anti-apoptosis via Inhibition of AIF Apoptogenic Effects but Not Caspase-3 Activation—Bacterial CpG DNA has been shown to induce dendritic cell survival via the PI3K pathway by preventing the cleavage of procaspase-3 and by down-regulating the formation of active caspase-3 fragments such as p17 (12). To evaluate whether CpG-B ODN-induced Hsp70 could have any influence on caspase-3 activity, we stably transfected RAW264.7 cells with antisense hsp70 cDNA (RAWHsp70as) and others with Hsp90β small interfering RNA (RAWHsp90βsRNAi) (Fig. 5A). Culture of cells in serum-deprived or STS-supplemented medium significantly increased caspase-3 activation (Fig. 5, B and C), which was modulated by CpG-B ODN but not GpC ODN (negative control). A similar inhibitory effect of CpG-B ODN on caspase-3 activation was found in RAWHsp70as cells (Fig. 5, B and C), indicating that CpG-B ODN does not depend on Hsp70 to inhibit caspase-3 activation. However, inhibition of Hsp90β by expressing Hsp90β small interfering RNA (RAWHsp90βsRNAi) significantly decreased the inhibitory effect of CpG-B ODN on caspase-3 activation (Fig. 5, B and C), suggesting that the modulating effect of CpG-B ODN on STS- or serum starvation-induced activation of caspase-3 likely depends on Hsp90β.

To further evaluate the involvement of Hsp70 in the anti-apoptotic effect of CpG-B ODN, we determined the effect of CpG-B ODN on AIF and investigated whether it could involve Hsp70. AIF is a caspase-independent death effector released from mitochondria and subsequently translocated into the nucleus in the apoptotic process (36). Much evidence has shown that AIF is essential for serum withdrawal- or STS-induced apoptosis (27, 37). Our results demonstrated that the amount of AIF contained in the nucleus of control cells was greatly increased after serum starvation (Fig. 6A) or STS treatment (Fig. 6B) and that this nuclear translocation of AIF was inhibited either by stable expression of Hsp70 in RAW264.7 cells (RAWHsp70as) or by CpG-B ODN treatment (Fig. 6, A and B). Moreover, the ability of CpG-B ODN to prevent AIF nuclear translocation was impaired in RAWHsp70as cells and quercetin-treated mouse pDCs (Fig. 6, A and B). Taken together, these results suggest that Hsp70 most likely participates in CpG-B ODN-mediated anti-apoptosis via preventing nuclear translocation of AIF.

Because the apoptogenic effect of AIF has been shown to be modulated by the cellular level of Bcl-xL (28), we then determined the relation between Hsp70 and Bcl-xL. Fig. 6 (C and D) shows that the Bcl-xL levels were increased in CpG-B ODN-treated mouse macrophages and RAW264.7 cells, but that the enhancement was inhibited in RAWHsp70as and quercetin-
Critical Role of Hsp70 in Anti-apoptosis of CpG-B ODN

DISCUSSION

It is well known that CpG-B ODN is capable of protecting B cells, macrophages, and pDCs against apoptosis (10–12). However, its mechanism of action remains to be further elucidated. Our study demonstrated for the first time that stimulation of macrophages and pDCs with CpG-B ODN but not GpC ODN for 14 h or longer resulted in up-regulation of Hsp70 via the TLR9/MyD88 pathway (Figs. 1 and 2) and that this increase in Hsp70 was positively associated with the anti-apoptotic effect of CpG-B ODN on cells cultured in serum-starved or STS-supplemented medium (Fig. 4). However, attenuation of Hsp70 expression by a synthesis inhibitor or antisense hsp70 cDNA interfered with CpG-B ODN-mediated anti-apoptosis (Figs. 3 and 4). These results thus suggest that the induction of Hsp70 by CpG-B ODN may contribute to the anti-apoptotic effect of CpG-B ODN.

Activation of the PI3K/Akt but not p38 MAPK pathway is believed to be positively associated with anti-apoptosis (12). Recently, in studying the relation between heat shock proteins and hypoxia-induced apoptosis, Zhou et al. (34) reported that PI3K/Akt contributes to stabilization of hypoxia-inducible factor 1α by provoking expression of heat shock proteins. Our findings that CpG-B ODN up-regulated Hsp70 primarily via the PI3K pathway (Fig. 2, D and E) and that inhibition of PI3K but not p38 MAPK modulated CpG-B ODN-mediated anti-apoptosis (Fig. 3D) provide further evidence for the importance of Hsp70 and PI3K in anti-apoptosis.

Although CpG-B DNA has been shown to prevent cell death via inhibition of caspase-3 activation (12), this study revealed that AIF nuclear translocation, a caspase-independent process, was involved in CpG-B ODN-mediated anti-apoptosis (Fig. 6). The treated mouse macrophages, suggesting that Hsp70 may be positively associated with the anti-apoptotic effect of CpG-B ODN via the Bcl-xL/AIF pathway.

FIGURE 4. Inhibition of Hsp70 by expression of antisense hsp70 cDNA decreases the anti-apoptotic effect of CpG-B ODN. A, RAW264.7 and pcDNA3.1(-)Hsp70 (pcDNA(empt))-transfected RAW264.7 cells were cultured for 60 h in serum (10% FBS) or serum-free medium (0% FBS) with or without CpG-B ODN (1 μM) in the presence or absence of 5 μM quercetin (Quer.) as indicated. B, RAW264.7 and pcDNA3.1(-)Hsp70AS (pcDNA(empt))-transfected RAW264.7 cells were incubated with CpG-B ODN for 14 h and then stimulated with STS for 8 h. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to determine cell viability. Data represent the means ± S.D. of at least three independent experiments. *, p < 0.05 for the decrease induced by quercetin or pcDNA3.1(-)Hsp70AS versus CpG-B ODN; **, p < 0.05 for the increase induced by pcDNA3.1(-)Hsp70 versus quercetin in the presence of CpG-B ODN.

FIGURE 5. Effect of inhibition of Hsp70 on CpG-B ODN-mediated deactivation of caspase-3. A, lysates of RAW264.7, RAWHsp70, and RAWHsp90AS cells or RAW264.7 cells stably transfected with Hsp90α small interfering RNA (RAWHsp90(empt)) were subjected to Western blot analysis with antibodies to Hsp70, Hsc70, Hsp90α, and actin. B, cells were cultured for 60 h in serum (10% FBS) or serum-free medium (0% FBS) with or without 1 μM CpG-B ODN or GpC ODN. C, cells were pretreated with CpG-B ODN or GpC ODN for 14 h, followed by STS stimulation for 8 h. The caspase-3 activity was measured using a fluorogenic substrate as described under “Experimental Procedures.” Data represent the means ± S.D. of at least two independent experiments. *, p < 0.05.
Critical Role of Hsp70 in Anti-apoptosis of CpG-B ODN

FIGURE 6. Effect of inhibition of Hsp70 on subcellular distribution of AIF and Bcl-xL expression. A, RAW264.7, RAWHsp70, and RAWHsp70AS cells and mouse pDCs were cultured for 60 h in serum (10% FBS) or serum-free medium (0% FBS) with or without CpG-B ODN for 14 h and then stimulated with STS for 8 h. The subcellular distribution of AIF in the nucleus (N) and cytosol (C) was analyzed by immunoblotting with an anti-AIF antibody. Mouse spleen macrophage cells were cultured in medium with or without CpG-B ODN (1 μM) for 16 h in the presence or absence of 5 μM quercetin as indicated. B, RAW264.7, RAWHsp70, and RAWHsp70AS cells were pretreated with or without CpG-B ODN for 14 h and then stimulated with STS for 8 h. The subcellular distribution of AIF in the nucleus (N) or cytosol (C) was analyzed by immunoblotting with antibodies to Bcl-xL and actin. Data represent the means ± S.D. of two experiments.

impaired the ability of CpG-B ODN to prevent AIF nuclear translocation (Fig. 6) but not caspase-3 activation (Fig. 5) suggest that induction of Hsp70 by CpG-B ODN may selectively contribute to the effect of CpG-B ODN on AIF. Recently, Zhu et al. (38) reported that the brain neurons of postnatal day 9 male mice with moderate hypoxia-ischemia displayed a more pronounced translocation of AIF, whereas those of female mice displayed a stronger activation of caspase-3. The advantage of selectively inhibiting AIF translocation but not caspase-3 activation remains, however, to be elucidated.

Hsp70 has been shown to increase the expression and transcriptional activity of STAT5 (signal transducer and activator of transcription 5), thereby improving the anti-apoptotic activity of Bcl-xL (35) to inhibit caspase-3 activation and to prevent nuclear translocation of AIF (35, 39). Our experiments showed an increase in the level of Bcl-xL by CpG-B ODN, which was attenuated by inhibiting Hsp70 expression with an inhibitor or antisense Hsp70 (36). Thus, CpG-B ODN might induce the expression of Hsp70, which then activates Bcl-xL to block nuclear translocation of AIF, resulting in anti-apoptosis. Of note, we did not observe a significant decline in the inhibitory effect of CpG-B ODN on caspase-3 activation upon antisense Hsp70 expression in RAW264.7 macrophages. CpG-B ODN may thus modulate the activation of caspase-3 in these cells primarily via pathway(s) other than the induction of Hsp70. One of the likely mediators is Hsp90β, which has been shown to be up-regulated after CpG ODN treatment (40) and which is an excellent modulator of caspase-3 activation (Fig. 5, B and C). The influence of Hsp90β on the anti-apoptotic effect of CpG ODN has been revealed (supplemental Fig. 5), with further study being undertaken to address this issue.

In summary, our results demonstrate that CpG-B ODN up-regulates the expression of stress-inducible Hsp70 (without affecting constitutively expressed Hsp70) via a TLR9/MyD88/PI3K signaling pathway. The enhanced Hsp70 protein expression may be positively associated with CpG-B ODN-mediated anti-apoptosis primarily via increasing the level of Bcl-xL and inhibiting AIF translocation without activating caspase-3.

Acknowledgments—We thank Dr. D. M. Klinman for providing plasmid pcDNA-hTLR9 and Dr. Anke Klippel for kinase-deficient plasmid M-p110Δkin-myc.

REFERENCES
1. Krieg, A. M. (2002) Annu. Rev. Immunol. 20, 709–760
2. Barton, G. M., Kagan, J. C., and Medzhitov, R. (2006) Nat. Immunol. 7, 49–56
3. Ito, T., Wang, Y. H., and Liu, Y. J. (2005) Springer Semin. Immunopathol. 26, 221–229
4. Hacker, H., Mischak, H., Miethke, T., Liptay, S., Schmid, R., Sparwasser, T., Heeg, K., Lipford, G. B., and Wagner, H. (1998) EMBO J. 17, 6230–6240
5. Akira, S., and Takeda, K. (2004) Nat. Rev. Immunol. 4, 499–511
6. Liew, F. Y., Xu, D., Brint, E. K., and O’Neill, L. A. (2005) Nat. Rev. Immunol. 5, 446–458
7. Verthelyi, D., and Zeuner, R. A. (2003) Trends Immunol. 24, 519–522
8. Lenert, P., Rasmussen, W., Ashman, R. F., and Ballas, Z. K. (2003) DNA Cell Biol. 22, 621–631
9. Hartmann, G., Battiany, J., Pocek, H., Wagner, M., Kerkmann, M., Lube-now, N., Rothenfusser, S., and Endres, S. (2003) Eur. J. Immunol. 33, 1633–1641
10. Yi, A. K., Chang, M., Peckham, D. W., Krieg, A. M., and Ashman, R. F. (1998) J. Immunol. 160, 5989–5996
11. Yi, A. K., Hornbeck, P., Laffrenz, D. E., and Krieg, A. M. (1996) J. Immunol. 157, 4918–4925
12. Park, Y., Lee, S. W., and Sung, Y. C. (2002) J. Immunol. 168, 5–8
13. Sohn, W. J., Lee, K. W., Choi, S. Y., Chung, E., Lee, Y., Kim, T. Y., Lee, S. K., Choe, Y. K., Lee, J. H., Kim, D. S., and Kwon, H. J. (2006) Mol. Immunol. 43, 38206 JOURNAL OF BIOLOGICAL CHEMISTRY VOLUME 281 • NUMBER 50 • DECEMBER 15, 2006
Critical Role of Hsp70 in Anti-apoptosis of CpG-B ODN

1163–1171

14. Robert, J. (2003) *Dev. Comp. Immunol.* 27, 449–464

15. Volloch, V. Z., and Sherman, M. Y. (1999) *Oncogene* 18, 3648–3651

16. Garrido, C., Gurbuxani, S., Ravagnan, L., and Kroemer, G. (2001) *Biochem. Biophys. Res. Commun.* 286, 433–442

17. Tsuchiya, D., Hong, S., Matsumori, Y., Shiina, H., Kayama, T., Swanson, R. A., Dillman, W. H., Liu, J., Panter, S. S., and Weinstein, P. R. (2003) *J. Cereb. Blood Flow Metab.* 23, 718–727

18. Komarova, E. Y., Afanasyeva, E. A., Bulatova, M. M., Cheetham, M. E., Margulis, B. A., and Guzhova, I. V. (2004) *Cell Stress Chaperones* 9, 265–275

19. Park, K. C., Kim, D. S., Choi, H. O., Kim, K. H., Chung, J. H., Eun, H. C., Lee, J. S., and Seo, J. S. (2000) *Arch. Dermatol. Res.* 292, 482–487

20. Iordanskiy, S., Zhao, Y., Dubrovsky, L., Iordanskaya, T., Chen, M., Liang, D., and Bukrinsky, M. (2004) *J. Virol.* 78, 9697–9704

21. Beere, H. M. (2001) *Sci. STKE* 2001, re1

22. Jaattela, M., Wissing, D., Kokholm, K., Kallunki, T., and Egeblad, M. (1998) *EMBO J.* 17, 6124–6134

23. Joza, N., Susin, S. A., Daugas, E., Stanford, W. L., Cho, S. K., Li, C. Y., Sasaki, T., Elia, A. J., Cheng, H. Y., Ravagnan, L., Ferri, K. F., Zamzami, N., Wakeham, A., Hakem, R., Yoshida, H., Kong, Y. Y., Mak, T. W., Zuniga-Pflucker, J. C., Kroemer, G., and Penninger, J. M. (2001) *Nature* 410, 549–554

24. Zhou, J., Schmid, T., Frank, R., and Brune, B. (2004) *J. Biol. Chem.* 279, 13506–13513

25. Susin, S. A., Lorenzo, H. K., Zamzami, N., Marzo, I., Snow, B. E., Brothers, G. M., Mangion, J., Iacotot, E., Costantini, P., Loeffler, M., Penninger, J. M., and Kroemer, G. (1999) *Nature* 397, 441–446

26. Yang, F., Sigua, C., Mali, P., George, P., Fiskus, W., Scuto, A., Annavarapu, S., Mouttaki, A., Sondarva, G., Wei, S., Wu, J., Djeu, J., and Bhalla, K. (2005) *Blood* 105, 1246–1255

27. Ahmad-Nejad, P., Hacker, H., Rutz, M., Bauer, S., Vabulas, R. M., and Wagner, H. (2000) *J. Exp. Med.* 192, 595–600

28. Otera, H., Ohsakaya, S., Nagaura, Z., Ishihara, N., and Mihara, K. (2005) *EMBO J.* 24, 1375–1386

29. Kuo, C.-C., Lin, W.-T., Liang, C.-M., and Liang, S.-M. (2006) *Biochim. Biophys. Acta* 176, 5943–5949

30. Tada, H., Shiho, O., Kuroshima, K., Koyama, M., and Tsukamoto, K. (1998) *J. Immunol. Methods* 93, 157–165

31. Ahmad-Nejad, P., Hacker, H., Rutz, M., Bauer, S., Vabulas, R. M., and Wagner, H. (2000) *J. Exp. Med.* 192, 595–600

32. Hacker, H., Vabulas, R. M., Takeuchi, O., Hoshino, K., Akira, S., and Wagner, H. (2000) *J. Exp. Med.* 192, 595–600

33. Kuo, C.-C., Liang, C.-M., and Liang, S.-M. (2005) *J. Immunol.* 176, 5943–5949

34. Tada, H., Shiho, O., Kuroshima, K., Koyama, M., and Tsukamoto, K. (1998) *J. Exp. Med.* 192, 595–600

35. Fukao, T., and Koyasu, S. (2003) *Trends Immunol.* 24, 358–363

36. Zhou, J., Schmid, T., Frank, R., and Brune, B. (2004) *J. Biol. Chem.* 279, 13506–13513

37. Joza, N., Susin, S. A., Daugas, E., Stanford, W. L., Cho, S. K., Li, C. Y., Sasaki, T., Elia, A. J., Cheng, H. Y., Ravagnan, L., Ferri, K. F., Zamzami, N., Wakeham, A., Hakem, R., Yoshida, H., Kong, Y. Y., Mak, T. W., Zuniga-Pflucker, J. C., Kroemer, G., and Penninger, J. M. (2001) *Nature* 410, 549–554

38. Zhu, C., Xu, F., Wang, X., Shibata, M., Uchiyama, Y., Blomgren, K., and Håberg, H. (2006) *J. Neurochem.* 96, 1016–1027

39. Yin, W., Cao, G., Johndres, M. J., Signore, A. P., Luo, Y., Hickey, R. W., and Chen, J. (2006) *Neurobiol. Dis.* 21, 328–371

40. Kuo, C.-C., Kuo, C.-W., Liang, C.-M., and Liang, S.-M. (2005) *Proteomics* 5, 894–906