Flagellin Contamination of Recombinant Heat Shock Protein 70 Is Responsible for Its Activity on T Cells*§

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Zhiyong Ye† and Yunn-Hwen Gan‡§†

From the †Department of Biochemistry, ‡Immunology Program, Yong Loo Lin School of Medicine, National University of Singapore, Block MD7, #05-10, 8 Medical Drive, Singapore 117597, Singapore

Heat shock proteins (Hsp) 60 and 70 have been intensively studied for their ability to activate innate immunity. Heat shock proteins had been shown to induce the activation of dendritic cells, T cells, and B cells. However, the possible contamination of endotoxin in heat shock protein preparations makes their function as an activator of immune system ambiguous. Here, we examined the ability of bacterial Hsp60 and Hsp70 to activate Jurkat T cells and primary T cells. We found that Burkholderia pseudomallei Hsp70 and Mycobacterium tuberculosis Hsp70 could costimulate Jurkat T cells to make IL-2 and signal through TLR5. This costimulatory activity is not due to endotoxin or contaminants signaling via TLR2 nor TLR4. However, recombinant Hsp70 expressed in Escherichia coli ΔflIC strain completely lost its ability to costimulate T cells. Thus, the activation of T cells by recombinant Hsp70 is ascribed to flagellin contamination.

Although heat shock proteins 60 and 70 have been touted to be potent activators of the innate immune system (1–3), recent studies have urged caution in the interpretation of these results due to the possibility of bacterial contaminants in the recombinant protein preparations. Recombinant human Hsp70 and Hsp60 cleaned of endotoxin contamination failed to induce activation and cytokine secretion from antigen-presenting cells such as macrophages (4–6). There had also been reports on the ability of heat shock proteins to activate T cells and B cells. For example, human T cells were reported to directly respond to soluble human Hsp60 via TLR2 (7, 8). The same group also reported activation of B cells by human Hsp60 via the TLR4-MyD88 pathway (9). Lipopolysaccharide (LPS)-treated Hsp60s were shown to enhance antigen-specific murine T cell activation (10).

Besides mammalian Hsps, mycobacterial Hsp70 has been shown to stimulate the release of cytokines and chemokines from dendritic cells (11, 12). Furthermore, a mycobacterial Hsp70 fusion protein with ovalbumin was shown to elicit a CD4+ T-cell-independent, ovalbumin-specific cytotoxic T lymphocyte (CTL) response in mice (13). Harmala et al. (14) extended the finding by demonstrating that immunization with the fusion protein in mice was qualitatively and quantitatively superior to complete Freund’s adjuvant and LPS in eliciting antigen-specific CTL responses in vivo and the adjuvant effect of the fusion protein was not due to LPS contamination. However, the mechanism of the adjuvanticity of the fusion protein was not addressed.

To determine whether bacterial Hsps belonging to the 60 and 70 families have a direct effect on T cells as had been reported for mammalian Hsp60 (10), we examine the ability of mycobacterial Hsp65 and Hsp70, as well as Burkholderia pseudomallei Hsp60 and Hsp70 to activate the human Jurkat T cell line as well as primary CD4+ T cells. Only Hsp70s are found to enhance T cell activation. However, this effect is completely abrogated when the recombinant proteins are made in Escherichia coli with the flagellin gene deleted. Thus, the costimulatory effect of T cell activation by Hsp70 is due to the presence of flagellin contamination in the recombinant protein preparations.

EXPERIMENTAL PROCEDURES

Materials—LPS from E. coli 055:B5 (catalog number L4005), Pam3CSK4 (catalog code lip2l-pms), iomycin (catalog number I9657), and phorbol 12-myristate 13-acetate (catalog number P1585) were purchased from Sigma. Purified mouse anti-human CD3 monoclonal antibody (anti-CD3, catalog number 145-1096), and phorbol 12-myristate 13-acetate (catalog number P1585) were purchased from Sigma. Recombinant Mycobacterium tuberculosis Hsp70 kindly provided by Dr. Paul A. MacAry (National University of Singapore) was purchased from Lionex GmbH (Product code: MT-70kDa, batch: 04-1). The pcDNA3.1 myc/His vector inserted with full-length cDNA encoding human TLR2, TLR4, and TLR5 (pTLR2, pTLR4, and pTLR5) were kindly provided by Dr. Jinhua Lu (National University of Singapore).

Cell Cultures—Jurkat clone E6-1 (ATCC, TIB-152) and human embryonic kidney HEK 293T (ATCC, CRL-11268) cells were cultured in RPMI 1640 (Invitrogen) and DMEM (Sigma), respectively, supplemented with 10% heat-inactivated fetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel), 2 mM L-glutamine (Sigma), 100 units/ml penicillin, and 0.1 mg/ml streptomycin (Sigma). Cells were maintained at 37 °C in a humidified 5% CO2 incubator.

Recombinant Protein Purification—The full-length B. pseudomallei Hsp60 (BpHsp60), Hsp70 (BpHsp70), flagellin (Flg) genes and the full-length M. bovis Hsp65 (MbHsp65) gene

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with a His, tag at the N terminus were cloned into the expression vector pQE-30 (Qiagen) and transformed into E. coli M15 cells. The expression vector of BpHsp70 was also transformed into E. coli ΔflfC- strain NK9375 kindly provided by Dr. N. Kleckner (15). The recombinant proteins were expressed and purified as described previously (16).

Isolation of CD4+ T Cells from Human Peripheral Blood—Peripheral blood mononuclear cells were prepared from healthy blood donors using HISTOPAQUE®-1077 (Sigma). CD4+ T cells were isolated from peripheral blood mononuclear cells by magnetic cell sorting using human CD4+ microbeads (Miltenyi Biotec), according to manufacturer’s instruction. The purity of CD4+ T cells was over 98% as determined by flow cytometry via staining with CD3-PE and CD4-FITC (BD Pharmingen).

Transient Transfection of Cell Lines—HEK 293T cells were seeded in the 24-well plate 24 h prior to transfection. The cells were transfected with two luciferase reporter plasmids, p5xNF-κB-luc (Stratagene, La Jolla, CA) and pRL-CMV (Promega, Madison, WI), using the GenePorter 2 transfection reagent according to the manufacturer’s instructions (Gene Therapy System, La Jolla, CA). HEK 293T cells were also co-transfected with the pTLR5 plasmid. The total amount of DNA transfected was kept constant with the pcDNA empty vector. Jurkat cells were seeded at a density of 0.8 × 10⁶/ml in the 24-well plate and transfected with 0.25 μg of each of the luciferase reporter plasmids per well using TransIT-Jurkat transfection reagent according to the manufacturer’s instruction (Mirus, Madison, WI).

Determination of Relative NF-κB Activity—NF-κB activation was measured as previously described (17). Briefly, 24 h post-transfection, cells were incubated with LPS, Pam3CSK4, Flg or the recombinant Hsp60 protein for 6 h. The cells were lysed, and luciferase expression in the lysate was determined by the dual luciferase assay kit according to the manufacturer’s instructions (Promega). In each experiment, NF-κB-mediated luciferase expression was normalized to CMV-mediated constitutive luciferase expression and annotated as relative luciferase activity.

Determination of IL-2 Concentration—Jurkat or CD4+ T cells (0.2 × 10⁶/well) were incubated with LPS, Pam3CSK4, Flg, or the recombinant Hsps in 96-well MaxiSorp plate (Nunc), which was coated overnight with 100 μl of 2.5 μg/ml anti-CD3 antibody. After 20 h (24 h for CD4+ T cells), supernatants were collected and assayed for IL-2 production using OptEIA human IL-2 enzyme-linked immunosorbent assay set (BD Biosciences, San Diego, CA) according to manufacturer’s instructions.

Colony PCR—Single colony of E. coli strain M15 and NK9375 was picked and subjected to PCR using the primer pair specific for fliC gene (5′-3′): ttagggcaacgtttaaatag/gattaacctcggaca- gagac (1501-bp amplicon). One PCR cycle consisted of the following: 94 °C for 30 s, 55 °C for 45 s, and 72 °C for 1.5 min. The total cycle number was 30 with a final elongation step at 72 °C for 7 min. The PCR product was separated on a 1.2% agarose gel.

Reverse Transcriptase-PCR—Total RNA of Jurkat cells was isolated using TRIzol reagent (Invitrogen). Subsequently, 5 μg of RNA was reverse transcribed using 200 units of M-MLV reverse transcriptase (Promega). PCR was conducted using 1 μl of cDNA and 2.5 units of Taq polymerase (Promega). The primers used for amplification of TLR2, TLR4, and TLR5 were described previously (18). The PCR conditions consist of 30 cycles of the following: 94 °C for 30 s, 55 °C for 45 s, and 72 °C for 1 min, followed by a final elongation step at 72 °C for 5 min. The PCR products (715 bp for TLR2, 623 bp for TLR4, and 446 bp for TLR5) were separated on a 1.2% agarose gel.

RESULTS

Hsp70 but Not Hsp60 Species Enhance IL-2 Secretion in Jurkat Cells—To determine whether Hsp60 and Hsp70 proteins of bacterial origin could act directly on T cells, recombinant heat shock proteins were added to Jurkat clone E6-1 cells, which have their T cell receptors cross-linked by immobilized anti-CD3 antibody. Incubated with anti-CD3 antibody alone, Jurkat cells secreted IL-2 at basal level. IL-2 concentration in the supernatants displayed a dose-dependent increase with the addition of recombinant B. pseudomallei Hsp70 (BpHsp70) (Fig. 1A). However, recombinant B. pseudomallei Hsp60 (BpHsp60) and M. bovis Hsp65 (MbHsp65) did not enhance IL-2 secretion even up to the concentration of 20 μg/ml and 40 μg/ml respectively (Fig. 1, C and D). To determine whether the costimulatory effect on Jurkat cells is general for bacterial Hsp70, we tested the commercially available M. tuberculosis Hsp70 (MtHsp70). Addition of MtHsp70 at higher concentrations than that of BpHsp70 increased the IL-2 secretion in a dose-dependent manner (Fig. 1B).

Hsp70 Costimulates Jurkat Cells in a Manner Similar to Flagellin—Endotoxin is known to be a common and potent contaminant of heat shock proteins (19). It is necessary to be cautious about the possibility that the contaminants could activate the secretion of IL-2 in Jurkat cells. The recombinant heat shock proteins used in our studies were produced in E. coli and extensively treated with deoxycholate to minimize LPS contamination. However, they could still be contaminated by bacterial components. To determine whether common bacterial contaminants could costimulate Jurkat T cells, we added TLR2, TLR4, and TLR5 agonists to our assay. Addition of TLR2 agonist Pam3CSK4 and TLR4 agonist LPS even at a high concentration of 10 μg/ml failed to enhance IL-2 secretion, while the TLR5 agonist flagellin was able to costimulate Jurkat cells (Fig. 2A). The enhancement of IL-2 secretion was found at a wide range of concentration of flagellin, ranging from pg/ml to μg/ml (data not shown). We further examined the expression of TLR2, TLR4, and TLR5 mRNAs in Jurkat cells. Only TLR5 mRNA was expressed in Jurkat cells, whereas TLR2 and TLR4 were not detected at mRNA level using gene specific primers (Fig. 2B). The pcDNA plasmid inserted with complete coding sequence of TLR2, TLR4, and TLR5 was used as positive controls for the primers (Fig. 2B).

Hsp70 Activates NF-κB in a Manner Similar to Flagellin—We further examined the ability of TLR agonists and heat shock proteins to stimulate NF-κB activation in the luciferase reporter gene assay in Jurkat cells (Fig. 3). Flagellin but not Pam3CSK4 nor LPS activated the NF-κB promoter on the firefly luciferase reporter plasmid through endogenous TLRs of Jurkat cells (Fig. 3A). BpHsp70 activated the NF-κB promoter in a dose-depend-
ent manner (Fig. 3B), NF-κB activation was also induced by MtbHsp70 at the concentrations of micrograms/ml (Fig. 3C). However, there was no activation upon the addition of either BpHsp60 or MbHsp65 (data not shown). Thus, the Hsp70 species behaved similarly as flagellin in terms of inducing NF-κB activation.

Presence of Contaminating Flagellin in Recombinant Hsp70 Preparations—To determine whether the ability of Hsp70 to costimulate Jurkat cells is independent of the effect of possible contaminating flagellin even in minute amounts, we expressed BpHsp70 protein in the E. coli strain NK9375, whose fliC gene is in-frame deleted (15). The absence of fliC gene in NK9375 was confirmed in colony PCR using gene specific primers (Fig. 4A). To verify that BpHsp70 expressed in NK9375 (ffBpHsp70) was truly flagellin-free, we transfected HEK293T cells with pTLR5 plasmid or control vector pcDNA and incubated the cells with this protein. ffBpHsp70 was unable to activate NF-κB in HEK 293T cells overexpressing TLR5, whereas BpHsp70 and MtbHsp70 retained their effectiveness in NF-κB activation (Fig. 4B). This shows that the ability of Hsp70 to activate NF-κB through TLR5 is due to contaminating flagellin in the recombinant protein preparations.

Flagellin-free Hsp70 Loses its Ability to Costimulate Human T Cells—We next examined the ability of the flagellin-free (ff) Hsp70 to activate NF-κB and costimulate Jurkat cells. We found that ffBpHsp70 could not activate NF-κB promoter in Jurkat cells compared with BpHsp70 (Fig. 5A). The costimulation of Jurkat cells by BpHsp70 was also abrogated in ffBpHsp70 (Fig. 5B). ffBpHsp70 did not increase IL-2 secretion even at the concentration of 10 μg/ml (data not shown). Therefore, flagellin is the contaminant in Hsp70 proteins that is crucial in inducing IL-2 secretion and NF-κB activation in Jurkat cells. We next isolated CD4+ T cells from peripheral blood mononuclear cells and costimulated T cells in an anti-CD3-coated plate with flagellin, BpHsp70, or ffBpHsp70 for 24 h. The enhanced IL-2 production was found in cells costimulated by flagellin and BpHsp70 but not in those costimulated by ffBpHsp70 (Fig. 5C). The results were consistent with those found in Jurkat cells and verified the role of flagellin as the contaminant in Hsp70 to induce IL-2 secretion in human T cells.

Having shown that Hsp70 did not directly stimulate T cells, we also determined whether it was able to regulate the response of Jurkat cells to various TLR agonists. No difference in IL-2

**FIGURE 1.** Hsp70 but not Hsp60 enhances IL-2 secretion in Jurkat cells. Jurkat cells were incubated with 1 μg/ml of ionomycin (Iono) plus 10 ng/ml phorbol 12-myristate 13-acetate or plate-bound anti-CD3 antibody alone or in the presence of various concentrations of Hsp70s (A and B) or Hsp60s (C and D) for 20 h at 37 °C, after which the supernatants were collected for determination of IL-2 concentration by enzyme-linked immunosorbent assay. Each bar represents the mean of triplicates with error bars showing the standard deviation. One representative experiment out of three is shown.

**FIGURE 2.** Flagellin but not LPS nor Pam3CSK4 (Pam) costimulates Jurkat cells. A, Jurkat cells were incubated with plate-bound anti-CD3 antibody alone (RPMI) or in the presence of indicated amount of Pam3CSK4, LPS, and Fig. The supernatant was collected for determination of IL-2 concentration by enzyme-linked immunosorbent assay. B, the total mRNA was extracted from Jurkat cells and reverse transcribed. The cDNA of TLR2, TLR4, and TLR5 was amplified using gene specific primers (upper panel). The plasmids containing complete coding sequence of human TLR2, TLR4, or TLR5, respectively, were used as positive control (lower panel). M represents the 100-bp DNA ladder. Each bar represents the mean of triplicates with error bars showing the standard deviation. One representative experiment out of three is shown.
secretion was found between the stimulations of TLR agonists alone or in the presence of 1 or 5 μg/ml of flagellin free BpHsp70 (see the supplemental figure). This clearly showed that BpHsp70 did not modulate IL-2 production in the response of Jurkat cells to TLR agonists.

DISCUSSION

Heat shock proteins are molecular chaperones known for their participation in the assembly and disassembly of proteins (20). In addition, they have also been found to bind peptides and chaperone them for better presentation on antigen presenting cells to elicit peptide-specific CTL responses (21). What has been more controversial is the purported ability of heat shock proteins to activate antigen presenting cells directly akin to the action of cytokines. Several studies had shown that the activating ability of Hsp60 and Hsp70 was due to endotoxin contamination and the usual heat inactivation control of Hsp was invalid as it also abrogated the activity of low concentrations of LPS (4–6). Furthermore, human Hsp60 has been shown to bind LPS specifically in a saturable manner through the binding motif LKGK (22).

Besides the possibility of LPS contamination in Hsps, LPS-associated molecules as well as other PAMPs could also be a potential source of contamination.

Our results with the activation of T cells by Hsp60 and Hsp70 show that only bacterial Hsp70 is contaminated with flagellin but not Hsp60. This shows that intrinsic differences exist between Hsp70 and Hsp60 family proteins in their biochemical properties and their ability to bind flagellin. Commercial preparations of the MtbHsp70 also contain flagellin. We did not examine whether recombinant mammalian Hsp60 and Hsp70 also have an affinity for flagellin. However, as flagellin is very potent in activating TLR5, care has to be taken to remove even traces of flagellin before examining the activation on various cell types.
After producing our recombinant BpHsp70 in E. coli strain lacking flagellin, we completely abrogated the ability of the protein to activate NF-κB and costimulate Jurkat T cells. All the costimulatory activity could be ascribed to flagellin, in agreement with recent studies showing the ability of flagellin to costimulate Jurkat T cells and primary CD4+ T cells. Flagellin at nanogram levels was able to costimulate T cells to make IFN-γ (23) and IL-2 (24). In our studies, flagellin at picogram levels could efficiently costimulate Jurkat T cells to make IL-2. This raises a particular point of concern. In studies where Hsps were shown to enhance CTL differentiation and activity, antigen presenting cells such as dendritic cells were co-cultured with T cells in the presence of antigen and Hsps. Since human dendritic cells and T cells do not express TLR5, it is important to ensure that the T cell activity observed was not due to a costimulatory effect of flagellin-contaminated Hsp nor due to a direct stimulation of the dendritic cells through TLR5 by flagellin. Flagellin has been shown to mature human dendritic cells and stimulate their production of chemokines (25). The same caution has to be applied to studies involving immunization of Hsp proteins as adjuvants in mice because flagellin is known to be a potent adjuvant in vivo (26, 27).

This is not to discount the ability of Hsps to stimulate the innate immune system. MacAry et al. (12) had shown that mycobacterial Hsp70 could induce an intracellular calcium oscillation in dendritic cells not seen in the response to LPS or other TLR agonists. The same research group recently reported that mycobacterial Hsp70 signaled through the CCR5 chemokine receptor to stimulate dendritic cells (28). Others had expressed mammalian Hsp60 on the surface of eukaryotic cells and still showed activation of T cells (10). As the binding ability of Hsps has been expanded with our description of flagellin contamination, one still needs to be cautious about the possibility of Hsps binding to other contaminants of bacterial origin that may not signal through the classical TLR signaling pathway or to endogenous PAMPs. An endogenous PAMP that had been described is uric acid (29) or, more accurately, monosodium urate crystals (30).

Thus, our studies have shown for the first time that flagellin contamination of recombinant Hsp70 preparations could contribute to observed stimulatory effect of Hsp on immune cells. As flagellin is a protein molecule, the usual controls of boiling or treatment with proteinase K will also abrogate the activity of flagellin. To exclude the possibility of flagellin contamination, it is critical that recombinant proteins are produced in E. coli strains not expressing flagellin.

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