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Heat Shock Up-Regulates lmp2 and lmp7 and Enhances Presentation of Immunoproteasome-Dependent Epitopes

Margaret K. Callahan, Elizabeth A. Wohlfert, Antoine Ménoret, and Pramod K. Srivastava

The heat shock response is a canonical regulatory pathway by which cellular stressors such as heat and oxidative stress alter the expression of stress-responsive genes. Some of these stress-responsive genes (heat shock proteins and MHC class I (MHC I)-related chains) play a significant role in the immune system. In this study, we have investigated the impact of stimulating the heat shock response on genes involved in the MHC I presentation pathway. We report that two inducible subunits of the proteasome, lmp2 and lmp7, are transcriptionally up-regulated by heat shock in cells of mouse and human origin. Furthermore, heat-shocked cells show enhanced presentation of the immunoproteasome-dependent MHC I antigenic epitopes NP118–126 of lymphocytic choriomeningitis virus and E1B192–200 of adenovirus, but not immunoproteasome-independent epitopes such as tumor Ag AH1 and SV40 large T Ag epitope II223–231. These findings show a novel immunological sequel to the cellular response to stress that may play a key role during fever or other homeostatic perturbations.

The heat shock response is a well-characterized pathway by which cellular stressors are sensed leading to the up-regulation of a panel of genes including heat shock proteins (hsps) (1). Although the traditional hsps (e.g., hsp70, hsp90, etc) represent the proteins abundantly up-regulated by heat shock, other proteins not traditionally classified as hsps are also regulated by this stimulus (2, 3). Underlying a role for stressors in modulating immune response, several immunologically relevant proteins are induced by heat shock including hsps, MHC class I (MHC I)-related chains A and B, and ubiquitin (4–6).

The endogenous Ag presentation pathway guides the generation, trafficking, and presentation of MHC I-restricted epitopes derived from cellular proteins. The whole protein is degraded in the cytosol by proteasomes (7), which are multisubunit structures with proteolytic activity restricted to a 20S core. The 20S core is shaped like a barrel with four rings of seven subunits, each stacked on top of each other. The two inner rings are made up of β subunits (β1–β7) and the two outer rings are made up of α subunits (α1–α7) (8–11). Only three of the β subunits (two copies of each) are proteolytically active: δ (β1), X (β5), and Z (β5). In cells stimulated by the proinflammatory cytokine IFN-γ, the composition of the proteasome is altered such that the three active β subunits are replaced by inducible subunits: lmp2 (β1i), lmp7 (β5i), and mecl1 (β2i) (12–17). This modified proteasome is the immunoproteasome (18).

The immunoproteasome is more likely to generate peptides with hydrophobic and basic C-terminal residues and less likely to generate peptides with acidic C-terminal residues (17, 19, 20). A number of antigenic epitopes are differentially processed by immunoproteasome-expressing cells. Two epitopes that have been well characterized to be preferentially presented in immunoproteasome-expressing cells are the Ld-restricted lymphocyte choriomeningitis virus (LCMV) nucleoprotein (NP)118–126 epitope and the Db-restricted adenovirus E1B192–200 epitope (21, 22).

In this study, we have explored the impact of heat shock on regulation of the MHC I presentation pathway. We show that heat shock induces the expression of proteasome subunits lmp2 and lmp7 but not mecl1, and enhances the presentation of the immunoproteasome-dependent but not other epitopes. In these characteristics, heat shock acts in a manner analogous to IFN-γ.

Materials and Methods

Reagents

Recombinant IFN-γ was purchased from Pierce. The following Abs were used: anti-actin clone AC40 (Sigma-Aldrich), rabbit antisera to lmp2 and lmp7 (Affinity BioReagents), anti-hsp70 SPA810 (Stressgen), anti-LCMV NP clone 1.1.3 (provided by M. Buchmeier, The Scripps Research Institute, La Jolla, CA), and anti-adenovirus E1B from Oncogene Research Products.

Cells

CT26, a BALB/c murine colon carcinoma, and SW620, a human colon carcinoma line, were purchased from American Type Culture Collection. SVB6 is T Ag-transformed murine fibroblast cells line, obtained from S. Tevethia (Pennsylvania State University, Hershey, PA). SVB6 cells stably expressing adenovirus E1B and CT26 cells stably expressing the model Ag LCMV NP were generated by transfection.

T cells

Ag-specific T cell lines were developed for the following epitopes: LCMV-NP118–126 (RPQASGVYM), an Ld-restricted epitope; adenovirus E1B192–200 (VNIRNCCCY), a Db-restricted epitope; and T-Ag223–231 (CKGVNKEYL), a Db-restricted epitope. The anti-AH1-specific T cell line was provided by Dr. R. Binder (University of Connecticut School of Medicine, Farmington, CT). The anti-NP T cell line was generated by immunizing BALB/c mice. The anti-E1B and anti-T-Ag T cell lines were generated by co-immunizing C57BL/6 mice with peptide mixed 1:1 (v:v) with CFA. All experiments involving mice were approved by the Institutional Animal Care and Use Committee of University of Connecticut School of Medicine.
Semiquantitative RT-PCR
RNA was extracted using TRIzol reagent (Invitrogen Life Technologies). Purified RNA was treated with DNase I (Invitrogen Life Technologies) for 15 min at room temperature. Reverse transcription was performed using SuperScript II (Invitrogen Life Technologies) according to the manufacturer’s instructions.

Quantitative RT-PCR
Immunoproteasome subunit mRNA was quantified by real-time quantitative PCR (qPCR) using the Quantitect SYBR Green PCR kit (Qiagen) with the iCycler iQ Real-Time PCR Detection System (Bio-Rad). Specific primers and conditions for lmp2, lmp7, and mecl1 are shown in Table III. Relative target gene mRNA expression was normalized to β-actin mRNA.

Results
Up-regulation of immunoproteasome subunits by heat shock
Cells of the murine fibrosarcoma SVB6 were cultured at 37°C or heat shocked for 1 h at 42°C followed by 7 h of recovery. As a positive control, cells were cultured for 8 h in medium containing 100 U/ml mouse IFN-γ. IFN-γ is well known to up-regulate many

Table I. Sequences and characteristics of PCR primers used for the murine genes indicated

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**FIGURE 1.** Heat shock up-regulates expression of immunoproteasome subunits. A, SVB6 cells were cultured at 37°C or for 1 h at 42°C with a 7-h recovery at 37°C or treated with 100 U/ml mouse IFN-γ for 8 h. RNA was reverse transcribed and primers for indicated genes were used to amplify the cDNA pool. Titrated dilutions of cDNA (1, 1/10, and 1/100) were used to assess the linearity of amplification. B, SVB6 cells were cultured at 37°C or for 1 h at 42°C followed by 7 h of recovery. As a positive control, cells were cultured for 8 h in medium containing 100 U/ml mouse IFN-γ. IFN-γ is well known to up-regulate many
proteins in the MHC I presentation pathway, including immunoproteasome subunits. At the end of the culture, total RNA was collected and reverse transcribed into a cDNA pool that was used for specific amplification of indicated transcripts using primers (Table I). Genomic contamination was routinely ruled out by amplification of nonreverse transcribed samples as negative controls (data not shown).

As expected, heat shock caused up-regulation of hsp70 transcripts. The subunits lmp2 and lmp7 are poorly expressed in cells cultured at 37°C, but induced in heat-shocked cells by ~10-fold (Fig. 1A). In contrast, the third subunit, mecl1 (β2i) was not up-regulated by heat shock under these conditions. Transcript levels of the constitutive subunits δ (β1i) and X (β5i) remained unchanged by heat shock. Cells treated with IFN-γ up-regulated the expression of lmp2 (β1i) and lmp7 (β5i) by ~20- and 100-fold, respectively. Actin was used as a control for equivalent starting quantity.

To test whether the effect of heat shock on lmp2 and lmp7 transcripts translated into increased protein levels, heat-shocked SVB6 cells were assayed for lmp2 and lmp7 by immunoblotting. As compared with cells cultured at 37°C, heat-shocked cells expressed increased steady-state levels of lmp2 and lmp7 proteins; cells treated with IFN-γ had a more robust increase in expression of these subunits.

To explore the generality of this phenomenon, we tested it in CT26, a murine colon carcinoma, and SW620, a human colon carcinoma. The cells were cultured at 37 or at 42°C or treated with IFN-γ to induce or block heat shock conditions; extracted RNA was reverse transcribed to generate cDNA for qPCR analysis. Heat shock caused a 14.2-fold induction of lmp2, 20.8-fold induction of lmp7, and 1.5-fold induction of mecl1 (Fig. 1D). These data are consistent with the induction seen by gel analysis of semiquantitative PCR as shown in Fig. 1C. This pattern was also observed in the human tumor cell line SW620 (Fig. 1E). Expression of GAPDH was unaffected by heat shock or IFN-γ (Tables II and III).

### Immunoproteasome-dependent and -independent epitopes

Reagents were developed to study two immunoproteasome-dependent epitopes: adenoaviral E1B192–200 and LCMV NP118–126 (Table II). A T Ag-transformed fibroblast line, SVB6 (D3), was engineered to express adenoaviral protein E1B as described in Materials and Methods. Transfectants were cloned and screened and a high-expressing clone 315 was chosen (Fig. 2A). The T Ag, also expressed in these cells contains another Dα-restricted epitope, epitope Iα223–231, whose generation is not dependent on immunoproteasomes. In an independent system, CT26 cells, which express the Lα-restricted AH1 AG 23, were engineered to express the NP of the LCMV. Transfectants were cloned and the high NP-expressing clone C was chosen (Fig. 2D). The Lα-restricted NP118–126 epitope is preferentially generated in immunoproteasome-expressing cells (22). In contrast, the Lα-restricted AH1 epitope is immunoproteasome-independent.

T cell lines specific for the E1B192–200 epitope and the NP118–126 were generated (see Materials and Methods). The specificity of these T cell lines was verified in a series of experiments. The E1B-specific T cell line was tested to establish peptide specificity by comparing the responses to various stimulators including E1B Ag-negative cells (EL4), EL4 cells pulsed with the E1B192–200 epitope, and EL4 cells pulsed with a control peptide, T Ag epitope II. The E1B-specific T cell line secretes IFN-γ upon stimulation with EL4 (D3) cells pulsed with the E1B epitope, but not an irrelevant T Ag epitope (Fig. 2B). Next, we tested the ability of the E1B-specific T cell line to recognize endogenously processed and presented E1B Ag. In this case, we compared the ability of E1B Ag-expressing clone 315 cells to the parental, E1B Ag-negative, SVB6 cell line. The E1B-specific T cell line generated IFN-γ in response to clone 315, but not the untransfected parental SVB6 (compare the first and second bars in Fig. 2C). Thus, the E1B-specific T cell line is both specific and sensitive enough to detect endogenously processed E1B192–200 peptide. Finally, to confirm that this T cell line is recognizing the MHC I-E1B192–200 epitope complexes, we added a MHC blocking Ab, K44, to the mixture of T cell and stimulator cells. The addition of K44 Ab abrogates IFN-γ release (compare the second and third bars in Fig. 2C), whereas the addition of an isotype control Ab has no effect (compare the second and fourth bars in Fig. 2C), thus, confirming that activation of the E1B-specific T cell line is MHC I restricted (Fig. 2C). The specificity of a T cell line against NP118–126 was similarly characterized. For the NP-specific T cell line, we compared stimulation by the LCMV NP Ag-negative cell line (CT26) with CT26 pulsed with exogenous NP118–126 peptide or CT26 pulsed with a control peptide, AH1.

### Table II. Sequences and characteristics of PCR primers used for the human genes indicated

<table>
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<td>Lmp2 R</td>
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<tr>
<td>Hsp70 F</td>
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<tr>
<td>Hsp70 R</td>
<td>CTA GAA ACG GAA CAC TGG AT</td>
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</table>

<sup>t<sub>m</sub></sup>: Melting temperature.

<table>
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<td>Lmp7 F</td>
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<td>Lmp7 R</td>
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<tr>
<td>Mecl1 F</td>
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<tr>
<td>Mecl1 R</td>
<td>GTG ATC ACA CAG GCA TCC AC</td>
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NP-specific T cell line responded to cells pulsed with the correct peptide and not the irrelevant peptide (Fig. 2E). Furthermore, the epitope-specific response could be blocked by the MHC blocking Ab K44 but not an isotype control Ab (Fig. 2F). The activity of T cells was tested in the presence of MHC-blocking Ab K44 or an isotype control Ab. 

**FIGURE 2.** E1B- or LCMV NP-expressing cell lines and Ag-specific T cell lines. A, SVB6 cells or SVB6 cells stably transfected with plasmid-expressing adenovirus E1B protein (clone 315) or HEK293 cells (which express E1B, as a positive control) were stained intracellularly with anti-E1B Ab. Isotype control-stained cells are represented by a dashed line. B, T cells specific for adenovirus E1B192–200 were stimulated with no cells, EL4 cells, EL4 cells pulsed with E1B192–200 peptide, or EL4 cells pulsed with T Ag223–231 peptide. T cells stimulation was evaluated by concentration of IFN-γ in culture supernatant. C, The activity of T cells was tested in the presence of MHC-blocking Ab K44 or an isotype control Ab. D, CT26 or CT26 stably transfected with plasmid-expressing LCMV NP (clone C) were stained intracellularly with anti-NP Ab. Isotype control-stained cells are represented by a dashed line. E and F, T cells specific for NP118–126 were stimulated with no cells, CT26 cells, CT26 cells pulsed with NP118–126 peptide, or CT26 pulsed with AH1 peptide. T cells stimulation was evaluated as in B. F, The activity of T cells was tested in the presence of MHC-blocking Ab K44 or an isotype control Ab.

NP-specific T cell line responded to cells pulsed with the correct peptide and not the irrelevant peptide (Fig. 2E). Furthermore, the epitope-specific response could be blocked by the MHC blocking Ab K44 but not an isotype control Ab (Fig. 2F).

**Enhanced presentation of immunoproteasome-dependent epitopes in heat-shocked cells**

Clone 315 cells were cultured at 37 or 42°C or treated with IFN-γ and each population was used to stimulate the E1B192–200-specific T cells. Clone 315 cells cultured at 37°C have a modest ability to stimulate anti-E1B T cells, whereas heat-shocked or IFN-γ-treated clone 315 cells have a significantly enhanced ability to stimulate them (Fig. 3A). In three independent experiments, cells heat shocked at 42°C consistently showed 2- to 3-fold enhancement of T cell stimulation ($p < 0.005$; Fig. 3B). SVB6 cells or clone 315 cells did not make IFN-γ themselves (data not shown). The enhanced T cell stimulation by heat-shocked clone 315 cells could derive from enhanced presentation of the recognized epitope or through up-regulation of Ag or MHC expression, or nonspecific activation of T cells. Experiments were performed to test these possibilities. Heat shock was observed to not up-regulate the expression of Dα on clone 315 (Fig. 3C), nor did it alter the levels of
EIB protein (Fig. 3D). Furthermore, the activity of heat-shocked cells as well as nonheat-shocked clone 315 cells to stimulate anti-EIB T cells was comparably abolished in the presence of the MHC I blocking Ab K44 (Fig. 3E), arguing against a nonspecific stimulation of T cells by heat-shocked cells. In a second assay to measure nonspecific activation, heat-shocked and control SVB6 cells were pulsed with titrated concentrations of EIB peptide. If heat-shocked cells possessed Ag-nonspecific stimulatory ability, we would predict enhanced T cell activation by peptide-pulsed heat-shocked SVB6 cells. This was not observed (Fig. 3F).

Similar experiments were conducted with the LCMV-transfected clone C. Cells were cultured at 37 or 42°C or treated with IFN-γ and tested for their ability to stimulate the NP118–126-specific T cells. Heat shock and IFN-γ treatment enhanced the ability of clone C cells to stimulate the T cells (Fig. 4A and B). CT26 and clone C cells do not make IFN-γ (data not shown). We observed no differences in the levels of staining for Ld or intracellular NP between heat-shocked and control clone C cells (Fig. 4C, C and D).

Lack of enhanced presentation of immunoproteasome-independent epitopes in heat-shocked cells

In contrast to the immunoproteasome-dependent EIB192–200 and NP118–126 epitopes, the presentation of immunoproteasome-independent TAg epitope II223–231 and the AH1 epitopes was unaffected by heat shock, as T cells against these specific T cells were equally stimulated by untreated, heat-shocked, and IFN-γ-treated cells (Fig. 5A and B). In both systems, because the immunoproteasome-dependent and -independent Ags are restricted by the same alleles and expressed by the same clone, the differences observed between the two epitopes are specific to the immunoproteasome-dependent epitope and not the MHC molecule or other cell-associated changes.

Discussion

Up-regulation of Lmp2 and Lmp7 is a general consequence of heat shock, as shown here in two murine and one human cell line. This up-regulation has an immunological consequence in that heat-shocked cells, such as IFN-γ-treated cells, show enhanced presentation of immunoproteasome-dependent MHC I epitopes in two different antigenic systems. The effect is specific to such epitopes as heat shock and has no effect on presentation of immunoproteasome-independent epitopes. Kuckelkorn et al. (24) previously investigated the effects of heat shock on the composition of proteasomes and found no significant changes in composition of proteasome in cells exposed to heat shock. Differences in the design of our experiments explain the differences in our conclusions. In the previous study, cells were heat shocked for 25 min at 42°C followed by a 60-min recovery before analysis of proteasomes. In contrast, we heat-shocked cells for 1 h at 42°C followed by 7-h recovery and assessment of transcripts of immunoproteasome subunits. Alternatively, we heat-shocked cells daily for 3 days followed by assessment of protein levels of immunoproteasome subunits and changes in Ag presentation. Given our observations that immunoproteasome subunits are transcriptionally up-regulated in response to heat shock, it is reasonable that the Kuckelkorn study which focused on the short-term effects of heat shock (changes that
The incorporation of subunits into the proteasome occurs during proteasome assembly (25, 26), is cooperative and favors populations of proteasomes with all three immunosubunits (27, 28). Nevertheless, mixed proteasome populations exist in vivo (25). Importantly, the mecl1 subunit is not required for the efficient incorporation of lmp2 or lmp7 (29). Presentation of the NP118–126 Ld-restricted epitope is enhanced in cells expressing three immunosubunits or in cells coexpressing mecl1 and lmp2 (30). Our observations suggest that up-regulation of mecl1 is not necessary for enhanced processing of the two immunoproteasome-dependent epitopes that we have studied or in the tumor cell lines that we used. Alternatively, the constitutive levels of mecl1 protein or very modest levels of up-regulation may be sufficient for generation of immunoproteasomes incorporating all three immuno subunits.

The level of induction of immunoproteasome subunits in response to heat shock was observed to be generally lower than the level of induction by IFN-γ. This may suggest that heat-shocked cells have a relatively modest ability to generate immunoproteasome-dependent epitopes. However, a study of the relationship between the level of immunoproteasome induction and presentation of an immunoproteasome-dependent epitope showed that small changes in immunoproteasome expression cause significant changes in Ag processing (21). Thus, even the lower levels of induction of immunoproteasome subunits by heat shock have an effect on changing Ag presentation patterns, as indeed was shown in the present studies.
The generation of immunoproteasomes by heat shock, and its parallel with a similar effect of IFN-γ, may have a physiological nexus. Elevation of temperature and elaboration of IFN-γ and/or TNF are common and coordinated effects of bacterial and viral infections. Interestingly, all of these agents have been shown to mediate maturation of dendritic cells. Generation of immunoproteasomes by heat as well as IFN-γ may reflect redundant pathways of generation of immunoproteasome-dependent epitopes under these conditions, and may shed much-needed light on the immunology of fever.

Acknowledgments

We thank Michael Oldstone (The Scripps Research Institute, La Jolla, CA) for sharing the anti-NP118–126 CTL clone HD-8 used in preliminary experiments, Mar Perez (The Scripps Research Institute) for providing the LCMV NP plasmid, Michael Bachmeier (The Scripps Research Institute) for providing the anti-AH1 T (Salk Institute for Biological Sciences, La Jolla, CA) for providing the LCMV NP plasmid, Michael Buchmeier (The Scripps Research Institute) for providing the LCMV NP plasmid, Robert Binder for providing the anti-AH1 T cell line. The technical assistance of Roxana Pickering is gratefully acknowledged.

Disclosures

P.K.S. has a significant financial interest in Antigenics Inc., a sponsored research agreement with which company has partially supported the research described in this study.

References