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Inducible Heat Shock Protein 70 Promotes Myelin Autoantigen Presentation by the HLA Class II

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In this study, we investigated the role of the inducible form of heat shock protein 70 (hsp70) in the presentation of the major putative autoantigen in multiple sclerosis, myelin basic protein (MBP), in the context of appropriate MHC class II. By coimmunoprecipitation, we found that MBP is associated with hsp70 in APC in an ATP/ADP-dependent manner. Additionally, using confocal microscopy, hsp70 was detected in the endocytic pathway of APC, where it colocalized with MBP and HLA-DR. The immunodominant epitopes of MBP 85–99 and 80–99 were shown to bind selectively and specifically to hsp70 by surface plasmon resonance. The functional significance of MBP interaction with hsp70 was demonstrated by the detection of enhanced responses of an MBP-specific T cell hybridoma to MBP and MBP 80–99 with increasing levels of hsp70 and reduced responses when hsp70 expression was diminished within APC-expressing DRA*0101, DRB1*1501 (DR1501). However, when MBP 85–99 was used as the stimulus, T cell hybridoma responses were not enhanced by hsp70 overexpression within APC, suggesting that hsp70 contributes to Ag processing rather than Ag presentation. The importance of a direct association between MBP and hsp70 in the presentation pathways was demonstrated by enhanced efficacy of MBP presentation by APC transfected with a plasmid vector encoding a fusion hsp70-MBP protein. This is the first report on the involvement of self-inducible hsp70 in MHC class II-dependent autoantigen processing by APC. It implicates that aberrant self hsp expression may lead to the enhancement/modulation of autoimmune responses. The Journal of Immunology, 2004, 172: 202–213.

A role for heat shock proteins (hsp)† as facilitators of immune responses to proteins and peptides has now been widely documented both in vivo and in vitro (1–5). The suggestion that hsp-associated epitopes are immunogenic, whereas the peptides alone are not, was first provided by Udono and Srivastava (6). They showed that an hsp70 preparation derived from cancer cells and containing cancer-specific peptides induced strong CTL response against peptides chaperoned by hsp (7–11). Most of these studies demonstrated the generation of a CTL response that was restricted by MHC class I molecules. Although the mechanisms involved in the enhanced immunogenicity of hsp-peptide complexes have not been fully delineated, recent studies suggest that hsp facilitate uptake of small amounts of peptide by APC via the common receptor CD91 that interacts with most classes of hsp (12, 13). Additional receptors for hsp that have recently been characterized on APC include Toll receptor 4 for hsp60 (14) and CD36 for gp96 (15).

Several studies supported the notion that hsp are also integrally involved in intracellular Ag presentation pathways. Transfection of hsp70 was shown to be able to rescue presentation of tumor Ags by MHC class I molecules in tumor cell lines (16). Hsp90 and hsp70 also have been shown to associate with TAP and other molecules of the MHC class I presentation pathway (17, 18). Treatment of APC with deoxyspergualin, an inhibitor of hsp70 and hsp90 (19, 20), significantly reduced their potency to present MHC class I-associated Ags to T cells (21). Although the majority of reports on the enhancing effect of hsp on Ag presentation address the MHC class I Ag presentation pathway, more recent studies have also implicated the hsp70 family in MHC class II presentation. Thus, hsp70 cognate form (hsc70) was recently identified as a cytosolic partner capable of interacting with the MHC class II invariant chain (II), as well as being responsible for the enlargement of the endocytic compartments (22). Furthermore, a dominant-negative version of hsc70 counteracted the ability of II to modify the endocytic pathway, demonstrating an interaction in vivo of II with hsc70 (22). However, the exact role of hsp70, the inducible form of hsp70 in the pathway of the MHC class II Ag presentation and as a potential chaperonin for MHC class II-associated Ags, remains to be addressed.

In this study, we investigated the involvement of hsp70 expression in the presentation of myelin basic protein (MBP) and MBP-derived peptides in an MHC class II-dependent manner. MBP represents one of the most immunogenic proteins of the CNS and is...
the putative autoantigen in multiple sclerosis (MS) (23, 24). Abundant expression of hsp has been noted in the pathologic lesions of MS (25–27). We now show that hsp70 overexpression significantly contributes to T cell recognition of MBP or an elongated MBP peptide that requires endosomal processing. We propose that aberrant hsp70 expression might contribute to recognition of auto-antigens and modulation of autoimmune responses.

Materials and Methods

Cells and reagents

MHC class II-restricted MBP presentation was tested using MBP-specific CD4+ T cell hybridomas that recognize MBP peptide 85–99 in the context of HLA-DR1501 (L1501). The hybridomas were generated from a human autoreactive CD4+ T cell clone, derived from a DR1501 homozygous MS patient, recognizing MBP peptide 85–99. The TCR α and β full-length cDNA were generated from RNA extracted from the clone. The TCR α and β expression vectors (pRep7-TCRα and pRep8-TCRβ) were generated by incorporation of TCR α and β cDNAs into pRep7 and pRep8 vectors (Invitrogen, Carlsbad, CA). TCR-negative Jurkat J.RT3-T3.5 hybridoma cells (American Type Culture Collection, Manassas, VA) were cotransfected with pRep7-TCRα and pRep8-TCRβ using Lipofectamine Plus reagents (Life Technologies, Gaithersburg, MD), according to the manufacturer’s instructions. Stable transfectant lines were generated by selection with hygromycin B (Invitrogen), and positively transduced hybridomas were selected by single cell cloning. Several lines were confirmed to express both TCR α and β by flow cytometry using FITC-labeled anti-human TCR αβ T109.1A.31 Ab (BD PharMingen, San Diego, CA). Because all selected lines expressed equal levels of TCR, as well as identical TCR recognition profiles, one of them was selected for subsequent experiments (data not shown).

For the presentation assays, a native murine fibroblast cell line (L cells) transfected with DR1501 (L1501) was used as the APC. L cells were shown to express functional HLA-DM and Ii and to be able to process and present MHC class II-associated Ags (28). Hsp70-inducible form, hsp70.1, hsp40, and MBP full-length cDNAs were generated from a murine spleen cDNA library using specific primers. Hsp70-MBP and hsp40-MBP fusion protein cDNA were generated by ligation of hsp70 cDNA or hsp40 cDNA and MBP cDNA, respectively, such that MBP was linked directly to the 3′ end of hsp70 or hsp40. Protein expression vectors were generated by the incorporation of the full-length cDNA into the expression vector pVax (Invitrogen), according to the manufacturer’s instructions. Green fluores- cence protein (GFP) full-length cDNA (Clontech Laboratories, Palo Alto, CA) was incorporated into the pVax vector to generate pVax-GFP expression vector. Empty linearized pVax vector was used as a control for the mock transfection experiments.

Hybridoma MBP presentation and DR inhibition assays

To exclude the possibility of autopresentation of MBP in the context of self MHC class II molecules by the human T cell clones, the MBP-reactive human TCR-derived T cell hybridomas were used for the presentation assays. T hybridoma cells (5 × 10^5/well) were cocultured with 5 × 10^5/well L1501 or native L cells in round-bottom 96-well plates. Ags were given in serial dilutions, and the total well volume was 200 µl. Presentation was allowed to occur for 24 h in culture, following which 100 µl of supernatant was harvested and transferred to a fresh plate for measurements of IL-2. The concentration of IL-2 in the supernatant was measured using the IL-2-specific HT.2 cells line by coinubation of supernatants with 5 × 10^5/well HT.2 cells, followed by measurement of HT.2 proliferation using [3H]thymidine incorporation over 18 h (29). The data represent Δcpm calculated by subtracting the background proliferation of unstimulated HT.2 cells. All presented values are means of triplicate cultures, and all experiments were performed four to six times. The requirement for MHC class II in the presentation assays was demonstrated using an anti-DR inhibition assay. For this assay, DR-blocking Ab (clone L243; BD PharMingen) was added at a concentration of 10 µg/ml at the initiation of the hybridomas and APC cocultures (30). Presentation was allowed to occur for 24 h in culture, following which 100 µl of supernatant was harvested and assayed for the presence of IL-2, as described above.

Hsp70 overexpression

Hsp70 overexpression was achieved by transfection of the fibroblastic murine cell line expressing DR1501 (L1501) with full-length murine hsp70.1 cDNA expression vector (pVax-hsp70). The transfection was performed using Lipofectamine Plus reagents (Life Technologies), according to the manufacturer’s instructions. The kinetics and efficacy of hsp70 overexpression were tested by Western blot analysis of L1501 cell lysates using anti-hsp70 mAb sc24 (Santa Cruz Biotechnology, Santa Cruz, CA). Because the sc24 Ab recognizes both hsp70 and hsc70, this allowed us to simultaneously control expression of both forms of hsp70. As a control, cells were transfected with empty pVax expression vector. No difference in HLA class II expression levels between hsp70 and mock-transfected cell lines, as determined by flow cytometry, using FITC-labeled anti-HLA-DR mAb TU36 (BD PharMingen). The efficiency of the pVax-hsp70 transient transfection was assessed in the parallel pVax-GFP transfection. The percentage of FL1-positive cells was ~75%, as assayed by flow cytometry (data not shown).

Hsp70 down-regulation

Hsp70 down-regulation was achieved in L1501 cells by infection with hsp70 antisense sequence containing Ad.ashp70 adenovirus. Subconfluent cells plated 24 h earlier were infected with adenovirus for 15 min at 25°C with continuous rocking, followed by 60 min at 37°C in RPMI 1640. The concentrations of virus used were optimized to cause 100% infection in each cell line without visible toxic effects according to the published protocol (31). Ad.Ahashp70 adenovirus was kindly provided by M. Jäättelä (University of Copenhagen, Copenhagen, Denmark). The efficacy of hsp70 down-regulation was tested by Western blot analysis of L1501 cell lysates using anti-hsp70 mAb sc24 (Santa Cruz Biotechnology) after several time points from 24 to 96 h postinfection, and the 60-h time point was selected for additional experiments.

Hsp70-MBP and hsp40-MBP fusion protein expression

Hsp70-MBP and hsp40-MBP fusion protein expression were achieved by transfection of the fibroblastic murine cell line expressing DR1501 (L1501) with full-length murine MBP cDNA sequence linked with either full-length murine hsp70.1 cDNA sequence or full-length murine hsp40 cDNA sequence in the expression vectors (pVax-hsp70-MBP or pVax-hsp40-MBP, respectively). Hsp40 is another member of the hsp family that was shown to be an important hsp70 co-chaperone (32). The efficacy and kinetics of the fusion protein hsp70-MBP expression were tested by Western blot analysis of L1501 cell lysates using anti-hsp70 mAb sc24 (Santa Cruz Biotechnology), as described above. Control cells were transfected with empty pVax expression vector. For the in vitro expression of MBP protein, L1501 cells were transfected with full-length murine MBP cDNA expression vector (pVax-MBP). The efficiency of MBP, hsp70-MBP, and fusion hsp40-MBP protein expression was assessed by Western blot analysis using anti-MBP mAb (Chemicon, Temacula, CA). The amounts of MBP and MBP fusion protein expression vectors used for transfections were optimized to assure similar levels of MBP and MBP fusion protein synthesis in L1501 cells (see Fig. 6B). Transfected cells were grown in 10% FCS until hsp70 expression was confirmed by Western blot. No difference in HLA class II expression levels was detected between hsp70-MBP, hsp40-MBP, MBP, and mock-transfected cell lines, as determined by flow cytometry using FITC-labeled anti-HLA-DR mAb TU36 (BD PharMingen). The efficiency of the pVax-MBP, pVax-hsp70-MBP, and pVax-hsp40-MBP transient transfections was assessed in the parallel pVax-GFP transfection. The percentages of FL1-positive cells were ~75%, as assayed by flow cytometry (data not shown).

Hsp70 peptide-binding assay by surface plasmon resonance

Bacterial hsp70 analog (DnaK; kind gift from M. Zylicz, International Institute of Molecular and Cell Biology) was immobilized on the surface of a CM5 sensorchip (Biacore AB, Uppsala, Sweden) at 40 µg/ml in 100 mM sodium acetate (pH 4.0). Between 5000 and 8000 resonance units were injected onto the sensor. To test ATP/ADP dependence of hsp70 interactions with peptides, the hsp70-MBP coupled sensorchip surface was washed with HBS supplemented with either 1 mM ADP or 1 mM ATP (Sigma-Aldrich) for 30 min at a flow rate of 30 µl/min, at 37°C. ATP was included in the last 5 min in the presence of MBP or MBP peptide. The efficiency of the ATP-dependent MBP binding was ~75%, as assayed by flow cytometry (data not shown).
Heat shock-induced up-regulation of hsp70 expression in APCs

Splenocytes were isolated from naive, female SJL/J mice, 6–8 wk of age, and plated on 96-well round-bottom plates and used as murine APCs. The cells were heat shocked for 1 h at either 40°C or 42°C, and the up-regulation of hsp70 was tested by Western blot analysis of splenocyte lysates using the anti-hsp70 mAb sc24 (Santa Cruz Biotechnology). The analysis of hsp70 expression was done at different time points after the start of heat shock (0, 10, 30, and 60 min).

MBP T cell line generation and proliferation assay

Female SJL/J mice, 6–8 wk of age, were immunized with MBP protein in CFA. Each mouse received 0.25 ml of a mixture of 0.8 mg of MBP protein dissolved in 0.1 ml of double-distilled H2O and 0.75 mg of Mycobacterium tuberculosis, in 0.15 ml CFA; injected s.c. in two abdominal sites. On day 12, cervical and inguinal lymph node cells were isolated from immunized mice and plaqued on 24-well plates to generate MBP-specific T cell lines. The cultures were stimulated on the day of isolation with MBP protein at 10 μg/ml, and on day 5 given murine IL-2 (10 μ/ml; PeproTech, Rocky Hill, NJ) and Con A (2 μg/ml; Sigma-Aldrich). Lymp node cell cultures were subsequently stimulated with MBP and IL-2 for another two rounds to generate MBP-specific T cell lines.

After the initial heat shock at 42°C for 1 h, splenocytes (see above) were pulsed with MBP at 1, 10, and 50 μg/ml and used for the proliferation assay as APCs. These APCs were added to MBP T cell lines in 96-well round-bottom plates (2 × 105 APCs and 106 T cell line cells/well) for 24 h and cultured at 40°C. Heating conditions and duration of the cultures were determined in several experiments to achieve clear up-regulation of hsp70 and to avoid nonspecific cell death due to high temperature, which was observed after 48 h of culture (data not shown). Cultures were pulsed with [3H]thymidine, 1 μCi/ml, for the last 14 h of the assay. Cells were harvested and [3H]thymidine incorporation was determined in a Wallac liquid scintillation counter (PerkinElmer Life Sciences, Wellesley, MA). As controls, we used cultures of nonheat-treated APCs. The data represent mean ± SEM calculated by subtracting the background proliferation of unpulsed cultures of APCs and T cell lines. All values shown represent the means of triplicate cultures, and experiments were performed three to four times.

Statistical analysis

Multifactor ANOVA test or Student’s t test was applied where appropriate to test significant differences in the IL-2 secretion assays and proliferation assay. Five percent (two tailed) was chosen as the level of significance.

Results

Hsp70 associates with MBP protein in the APC

To determine whether hsp70 associates with Ag in APC, we incubated the L1501 cells with MBP for 1 h and then immunoprecipitated hsp70 from the cell lysates. To exclude the possibility of nonspecific interactions, the samples were incubated during lysis with an excess of BSA in the lysis buffer. Hsp70 and associated proteins were separated by SDS-PAGE, transferred to Immobilon P membrane, and probed with Abs specific for MBP (Fig. 1A). Immunoblot analysis with the MBP-specific Abs showed a 21-kDa band, which corresponded to full-length MBP protein, that coimmunoprecipitated with the hsp70 molecule (Fig. 1A, lane 1); and no MBP was immunoprecipitated using a control polyclonal goat IgG (Fig. 1A, lane 5) and no MBP was immunoprecipitated from cells not pulsed with MBP (Fig. 1A, lane 2). No difference in MBP coimmunoprecipitation was found when the cell surface was trypsinized before the immunoprecipitation, suggesting an intracellular origin of the MBP-hsp70 complexes (Fig. 1A, lane 4). The presence of MBP-hsp70 complexes was ATP dependent (Fig. 1B), which is characteristic for hsp70-chaperoning activity. Furthermore, the supernatants from the last wash before cell lysis were assayed for the presence of soluble MBP. Because no soluble MBP was found, it is unlikely that hsp70-MBP complexes were formed extracellularly (Fig. 1C). Finally, the specificity of hsp70-MBP complexes was tested by analysis of potential formation of hsp70 complex with a control, intracellular protein. No
Association of hsp70 with different MBP peptides

To investigate the significance of association between hsp70 and MBP, as well as MBP peptides, we used a BIAcore assay, which is based on surface plasmon resonance. Surface plasmon resonance detects molecular interactions, because there is a corresponding change in refractive index when a macromolecule in solution binds to a macromolecule immobilized on the sensorchip. Hsp70 and a control protein, BSA, were immobilized on the surface of the sensorchip, and binding of fluid-phase MBP peptides was measured (Fig. 2). We selected a panel of 11- to 22-aa-long peptides spanning the entire MBP molecule: Ac1–11, 38–59, 61–82, 85–99, 85–99, 85–99, and 148–162. The BIAcore assays were run at two different pH conditions: neutral (7.0) and acidic (5.5), to reflect possible interactions occurring in either intracellular or specifically endosomal compartments. Sensorgrams of MBP peptide-hsp70 interactions showed a significant, specific binding of the 85–99 and 80–99 MBP peptides to the hsp70 at neutral pH conditions (Fig. 2A), while 85–99, 80–99, and 61–82 peptides bound at acidic pH (Fig. 2B). In contrast, none of the tested peptides showed any interactions with the control immobilized protein, BSA, for both tested pH conditions. Furthermore, the control tested peptides, PLEKQHEKERKQELEGES and SKEQREPQLEGEHQKEK, did not show any significant binding to hsp70 (Fig. 2C). Thus, with the panel of MBP peptides tested and two unrelated, control peptides, hsp70 was shown to be able to interact with MBP peptides 85–99, 80–99, and 61–82, but not with any other tested peptides.

Because hsp70 is capable of binding MBP, as we have shown previously (34), as well as MBP peptides, we performed a competition assay to determine the specificity of the protein/peptide binding site. We preincubated the sensorchip surface-immobilized hsp70 with MBP peptides to saturate its binding capabilities and then tested whether the capacity to bind MBP was still present. Interestingly, incubation with either MBP peptide (85–99 or 80–99) at both acidic and neutral conditions led to prevention of MBP binding to hsp70 (Fig. 2D). This result suggests that the MBP 80–99 amino acid domain may be responsible for binding of MBP protein to hsp70.

The ATP/ADP dependence of the MBP peptide 85–99, 80–99, and 61–82 binding to hsp70 was tested by preincubating sensorchip surface-immobilized hsp70 with either ADP or ATP and subsequent analysis of the MBP peptide binding for both acidic and

**FIGURE 1.** A, Demonstration of the presence of MBP-hsp70 complexes in APC. L1501 cells were lysed and immunoprecipitated with anti-hsp70 goat IgG (lanes 2–4) or with matched goat IgG (lane 5). All samples were probed with anti-MBP mAb (lanes 1–5, lower panel), and after Ab stripping, with anti-hsp70 (lanes 1–5, upper panel). Lanes 3, 4, and 5, Represent L1501 cells that were incubated with MBP, while lane 2 represents unpulsed L1501 cells. Lane 4, Represents L1501 cells that were treated with trypsin after the MBP pulse, before the immunoprecipitation step.

Lane 1, Represents the detection of reference hsp70 and PKCζ, an abundant cytoplasmic protein, was detected (Fig. 1D). Thus, we have shown that MBP-hsp70 complexes can be detected in the APCs, suggesting the specific, selective self hsp70 involvement in the intracellular fate of MBP within the APCs.

Coimmunoprecipitation of hsp70 and PKCζ, an abundant cytoplasmic protein, was detected (Fig. 1D). Thus, we have shown that MBP-hsp70 complexes can be detected in the APCs, suggesting the specific, selective self hsp70 involvement in the intracellular fate of MBP within the APCs.
neutral pH conditions (Fig. 3). For all the tested peptides, ATP incubations abrogated their binding to hsp70 at both pH conditions, while ADP had no effect on these interactions. Thus, hsp70-specific, ATP-dependent peptide activity of the observed interactions was shown, confirming the specificity of the interaction of MBP peptides 85–99, 80–99, and 61–82 with hsp70.

Overexpression of hsp70 leads to the increased presentation of MBP protein and MBP peptide 80–99 by HLA class II

To analyze the role of hsp70 in Ag presentation in the context of HLA class II, we overexpressed hsp70 in the APCs by transient transfection with pVax-hsp70 vector. The transfection with pVax-hsp70 resulted...
in a significant overexpression of hsp70, with peak expression occurring 24 h after transfection (Fig. 4A). In contrast, no difference from the control 0-h time point was noted in cells harvested 24 h posttransfection with the pVax control (Fig. 4A). Furthermore, no difference was found with respect to the expression levels of hsc70, the cognate form of hsp70 (Fig. 4A). To ensure maximum overexpression of hsp70, L1501 cells were used for the MBP protein/peptide presentation assay to MBP-specific T cell hybridomas 24 h posttransfection with hsp70 or empty vector. We found no difference in T cell hybridoma responses to MBP peptide 85–99 (Fig. 4D) when presented by cells with hsp70 overexpression vs empty vector-transfected APCs. In contrast, the presentation of either the extended MBP peptide 80–99 (Fig. 4C) or full-length MBP (Fig. 4B) was significantly enhanced when hsp70 was overexpressed, p < 0.01 and p < 0.02, respectively. This result suggests that hsp70 plays a role in the Ag-processing pathway in APCs. This effect was also critically dependent on the presence of the MHC class II Ags because native, DR-negative L cells were unable to present either MBP protein (Fig. 4E) or MBP peptides (data not shown) to the T cell hybridomas, and a DR-blocking Ab (L243) abrogated the MBP protein recognition by hybridomas for both L1501 and L1501 with hsp70 overexpression (Fig. 4E), p < 0.02. Thus, the specificity of MHC class II presentation of MBP and MBP peptides associated with hsp70 was confirmed.

**Down-regulation of hsp70 leads to a decrease in MBP protein/peptide presentation in the context of HLA class II**

To confirm further the role of hsp70 in the presentation of HLA class II-associated MBP Ags, we specifically down-regulated expression of hsp70 in APCs by expression of hsp70 antisense using an adenoviral delivery system (Fig. 5A). Interestingly, while the presentation of both MBP peptides 85–99 and the N terminus extended 80–99 was not modified by down-regulation of hsp70 (Fig. 5, C and D), the presentation of the MBP protein by APCs to MBP-specific hybridomas was diminished (Fig. 5B), p < 0.02. These results correspond to the hsp70 overexpression data, suggesting the involvement of hsp70 in the MBP presentation, especially in the processing phase.

**Expression of hsp70-MBP fusion protein within the APCs leads to increased HLA class II-associated Ag presentation**

To analyze the importance of a direct association between hsp70 in HLA class II-associated Ag presentation, we transfected the APCs (L1501) with either an MBP expression vector, hsp70-MBP, hsp40-MBP fusion protein expression vectors, or a control empty vector. The transfection of hsp70-MBP fusion protein resulted in maximum levels of the fusion protein expression in 24 h posttransfection, with no influence on the hsp70 and hsc70 expression levels (Fig. 6A). Transfection conditions with MBP, hsp70-MBP, and
FIGURE 4. A, Western blot demonstration of hsp70 overexpression kinetics in L1501-transfected cells. L1501 cells were transfected with pVax-hsp70 expression vector, as described in Materials and Methods. The cells were lysed at various time points posttransfection, and expression of hsp70 and hsc70 was determined by Western blot with anti-hsp70 murine mAb (lanes 2–5). As a control, L1501 cells were transfected with empty pVax vector, and after 24 h cell lysates were made and probed with anti-hsp70 (lane 1). Lane 6, Represents m.w. markers.

B–D, The enhancing effect of hsp70 overexpression in APC on the presentation of MBP protein (B), p < 0.02; MBP-extended peptide 80–99 (C), p < 0.01; but not on MBP peptide 85–99 (D). As a control, L1501 cells were transfected with empty pVax vector (mock transfection).

E, MHC class II dependence of the MBP protein presentation by hsp70-overexpressed L1501 cells was tested using DR-negative L cells and anti-DR Ab. IL-2 production by T cell hybridomas is shown, as measured by incorporation of [3H]thymidine by the IL-2-dependent cell line HT.2 with background proliferation correction (Δcpm, as described in Materials and Methods, ±SD).
hsp40-MBP fusion proteins were adjusted to yield similar kinetics and efficiency of protein synthesis in the APCs, as judged by Western blot analysis using anti-MBP Abs (Fig. 6B) and densitometry. This allowed us to make a direct comparison of the effects of transfections of APCs with single or fusion proteins encoding vectors on HLA class II MBP Ag presentation. We found that MBP transfection of APCs resulted in significantly higher responses of MBP-specific T cell hybridomas in the absence of any additional exogenous Ag, p < 0.04. Furthermore, expression of hsp70-MBP fusion protein led to an even stronger response of the T cell hybridomas, p < 0.04, whereas expression of hsp40-MBP fusion protein elicited similar levels of hybridoma activation to MBP overexpression, suggesting the enhancing effect of hsp70 fusion to MBP results from the direct association of both proteins rather than a nonspecific extension of the MBP transcript. These data further supported the role of hsp70 in the presentation of MBP Ags from the direct association of both proteins rather than a nonspecific extension of the MBP transcript. These data further supported the role of hsp70 in the presentation of MBP Ags in the context of HLA class II, suggesting an important role of direct association of hsp70 with MBP during Ag processing.

**Hsp70 subcellularly colocalizes with endosomal markers as well as with HLA-DR and internalized MBP**

To track the possible route of hsp70 action on the HLA-DR presentation pathway, we localized intracellular hsp70 with respect to the possible colocalization within the compartments critical for HLA-DR function (Fig. 7). Because the major site of Ag processing as well as loading of HLA-DR with peptides occurs in endosomes, we looked first for the presence of hsp70 within these structures. Transferrin, which binds transferring receptor in clathrin-coated pits, was used as a selective marker for endosomal trafficking. FITC-coupled transferrin was incubated with L1501 cells for 30 min and visualized by confocal microscopy. A vast majority of transferrin-positive structures were also positive for the hsp70 (Fig. 7A). Also, staining with the fluorophore-labeled Ab against a marker for early endosomes, EEA1, also revealed significant colocalization with hsp70 (Fig. 7D). Thus, hsp70 is present in the endosomal compartment as early as early endosome stage. Additional staining of L1501 cells with Ab against HLA-DR (Fig. 7B) and, following a pulse with MBP (Fig. 7C), with Ab against MBP revealed evidence for subcellular colocalization of hsp70 and both HLA-DR and internalized MBP. These data suggest that the presence of hsp70 in the endosomes permits direct access to both HLA-DR and internalized MBP. Colocalization of hsp70 and MBP also confirms the results of the MBP/hsp70 coimmunoprecipitation experiments, supporting the notion of subcellular association of hsp70 with MBP in APC.
A

Western blot analysis of kinetics of hsp70, hsc70, and hsp40-MBP fusion protein expression in APC transfected with hsp70-MBP expression vectors detected with anti-hsp70 mAb, as described in Materials and Methods (lanes 2–6). Lane 1, Represents m.w. markers.

B

Western blot analysis of efficiency of MBP, hsp40-MBP, and hsp70-MBP fusion protein expression detected with anti-MBP mAb in APC 24 h posttransfection with MBP, hsp70-MBP, and hsp40-MBP expression vectors, as described in Materials and Methods (lanes 2–5). Lane 1, Represents m.w. markers.

C

The enhancing effect of hsp70-MBP fusion protein expression in APC on the HLA class II-dependent MBP protein presentation. L1501 cells were transfected with either pVax-MBP, pVax-hsp70-MBP, or pVax-hsp40-MBP expression vectors or empty pVax vector as a control (mock transfection) (*, pVax-MBP vs mock transfection, p < 0.04; **, pVax-hsp70-MBP vs pVax-MBP, p < 0.04). IL-2 production by T cell hybridomas is shown, as measured by incorporation of [3H]thymidine by the IL-2-dependent cell line HT.2 with background proliferation correction (Δcpm, as described in Materials and Methods, ±SD).

Heat shock-induced up-regulation of hsp70 is associated with enhanced MBP-induced T cell proliferation

In attempt to relate the results of genetically induced hsp70 overexpression using the hybridoma system with more physiologic hsp70 induction, we analyzed the potential influence of hsp up-regulation resulted from heat shock on the MBP presentation process. As a source of natural APCs, we used splenocytes of SJL/J mice, and as responder cells an MBP-specific murine T cell line. Up-regulation of hsp70 was apparent in heat-shocked murine splenocytes as early as 30-min incubation after temperature elevation (Fig. 8, A and B). Furthermore, the heat-induced up-regulation of hsp led to increased proliferative responses of MBP-specific T cell line (Fig. 8C). These results demonstrate the potential physiological relevance of stress-induced expression of hsp70 and autoantigen recognition.

Discussion

In this study, we have shown that the inducible form of hsp70 enhances MHC class II-dependent presentation of the myelin-derived autoantigen, MBP. Hsp70 enhanced T cell recognition of MBP, as well as an elongated MBP immunogenic peptide, but not a trimmed MBP peptide. We have also shown, using two independent techniques, coimmunoprecipitation and confocal microscopy, that hsp70 can associate with MBP within APC. Because hsp70 was also present in the endosomal compartment and colocalized with HLA-DR, it suggests that hsp70 may modulate MBP processing within the MHC class II presentation pathway.

It is now well accepted that hsp act to chaperone peptides present in the cytosol for presentation and processing via the MHC class I molecule-loading pathway (12, 35). It has also been shown that hsp-associated peptides can enter an acidic compartment and be loaded onto MHC class II molecules (36). Our results, using MHC class II APC with overexpressed hsp70 and an MBP-specific TCR hybridoma as well as T cell lines, demonstrate a role for hsp70 in the immune responses dependent on MHC class II. The dependence of MBP presentation on MHC class II expression was confirmed by the lack of presentation by APCs that had not been transfected with DR1501, and by abrogation of the response with an Ab that blocked MHC class II on the cell surface. In addition, hsp70 has been localized in the endocytic compartment. This is also the first demonstration to show that inducible self hsp70 enhances Ag presentation in an MHC class II-dependent manner. It has also not been shown previously that hsp can enhance presentation of endogenous proteins that can serve as an autoantigen. Recently, Roth et al. (37) demonstrated an enhancing effect of bacterial DnaK, the Escherichia coli analog of hsp70, in the MHC class II-dependent presentation of recombinant human acetylcholine receptor α subunit Ag. Panjwani et al. (38) demonstrated that overexpression of hsc73 in macrophages enhanced presentation of the exogenous Ags hen egg lysozyme and C5 protein to appropriate hybridoma T cells, and Matsutake and Srivastava (36) have implicated gp96 in MHC class II presentation. Inducible murine hsp70, although related to cognate forms of the protein as well as closely related to its bacterial homologue, DnaK, is specifically transcribed under different stress conditions and represents a typical self stress-induced molecule involved in the majority of inflammatory processes. Additionally, we have shown that inhibition of hsp70 resulted in the diminished capability of APC to present MBP. This observation underscores the important role of hsp70 in MBP presentation, but also indicates that hsp70 may not only enhance Ag presentation in a stress-inducing environment, but may also be involved in regular mechanisms controlling Ag presentation. In this regard, the hypothesis of close evolutionary similarities between hsp and MHC molecules serving as complementary mechanisms of Ag recognition seems to be of particular interest (1, 39).

We studied MBP, a putative autoantigen in the human autoimmune neurologic disease, MS. It has been reported by us and others (25–27, 40, 41) that hsp, including hsp70, can accumulate in...
the pathologic lesions of MS within the CNS tissue. Using in situ immunocytochemical and molecular techniques, it has been shown that accumulation of hsp70 occurs in glial cells implicated in Ag re-stimulation within the CNS (42). We have shown in this study, using surface plasmon resonance technique, that hsp70 is capable of binding both MBP and MBP peptides 85–99 and 80–99. Furthermore, the

![FIGURE 7. APC (L1501 cells) were formaldehyde fixed and Triton X-100 permeabilized and stained for immunofluorescence analysis of intracellular colocalization of hsp70. Cells were labeled with anti-hsp70 and Cy5-coupled anti-rabbit IgG (red; left panel). A. To label the endosome compartment, cells were allowed to internalize FITC-coupled transferrin (green; middle panel). B. To localize intracellular distribution of HLA-DR molecules, cells were stained with FITC-coupled anti-DR (green; middle panel). C. To visualize internalized MBP, cells were incubated with MBP, followed by staining with anti-MBP and FITC-coupled anti-mouse Ig (green; middle panel). D. Early endosome compartment was visualized by staining with FITC-coupled anti-EEA1 (green; middle panel). For all panels, superimpositions of Cy5 and FITC are represented in yellow (right panel).](image)

![FIGURE 8. Western blot (A) and densitometric (B) analysis of kinetics of hsp70 up-regulation following heat shock at 40°C and 42°C. C. SJL/J splenocytes were incubated for 1 h at 42°C, followed by pulsing with MBP, and used as APC for MBP-reactive T cell lines. The presentation was allowed to occur for 24 h in culture at 40°C. Controls were cultures with APCs that were nonheat treated (37°C). T cell proliferative responses are represented as Δcpm (as described in Materials and Methods, ± SD), p < 0.04.](image)
competitive binding assay revealed the importance of MBP 80–99 domain in MBP binding to hsp70. Such coincidence of hsp70 association with an immunodominant epitope of MBP makes feasible a scenario in which hsp overexpressed by inflammatory mediators within the CNS lesions may be responsible for enhanced myelin autoantigen presentation. This hypothesis would require further confirmation, but implicates aberrant hsp expression in the mechanisms involved in the propagation of inflammatory processes and autoimmunity.

Molecular mechanisms of hsp enhancement of Ag recognition within the cell are currently not known. We have shown that overexpression of hsp70 only facilitates recognition of the whole MBP molecule or the elongated peptide 80–99, but not the trimmed peptide, which can directly be loaded onto MHC class II molecules. These results strongly suggest that hsp70 is involved in MBP processing within APC. The length of the principal human HLA-DR2-restricted epitope, aa 85–99 of MBP that is recognized in connection with HLA class II DRB1*1501 is 15 aa (43, 44). Our results indicate that presentation of such a peptide (MBP 85–99) is not facilitated by hsp70, although it maintains hsp70-binding capabilities assessed by surface plasmon resonance. However, MBP peptide extended by 5 aa at the N-terminal side does require hsp70 for efficient presentation. Recent findings by Menoret et al. (45) indicate that gp96 participates in trimming of N-terminal-extended peptides, which are then processed in the lumen of the endoplasmic reticulum before MHC class I loading. They further showed that gp96 is an aminopeptidase that can trim 19-mer precursors of an octamer peptide derived from stomatitis virus to the ready-to-go octamer for MHC class I presentation. Our results suggest that hsp70 might serve a similar function for N-terminal-elongated peptides processed for MHC class II presentation. Alternatively, hsp70 can cooperate with other aminopeptidases in the endosomal compartments and protect the peptide from complete proteolysis, ensuring an appropriate peptide length for association with MHC class II molecules, and chaperone them to the site of loading onto MHC molecules (46). This possibility is supported by earlier findings that Abs to hsp70 inhibit MHC class II Ag presentation (47). Cooperation with other lysosomal enzymes seems feasible because hsp70 down-regulation had a greater impact on the presentation of the whole molecule of MBP than for MBP 80–99 peptide.

We have also demonstrated a direct association between MBP and hsp70 within APC by coimmunoprecipitation and by colocalization of these two proteins using confocal microscopy. The importance of this association was demonstrated in transfection experiments with vectors encoding either MBP protein or hsp70-MBP and hsp40-MBP fusion protein. Although transfections with MBP and hsp70-MBP fusion protein yielded similar kinetics and efficiency of MBP synthesis in the APCs, a significantly higher response to APC-expressing fusion protein was detected with the hybridoma cell line. Furthermore, coupling of MBP to hsp40, another member of a closely related hsp family and an important hsp70 chaperone (32), by expression of hsp40-MBP fusion protein expression in APCs did not lead to MBP recognition beyond the point of MBP expression alone. These results might indicate the importance of direct interactions between MBP and hsp70 proteins rather then indirect associations, through secondary cochaperones, on to the endosomal/lysosomal compartment, representing the early phase of the Ag presentation pathway. Lysosomal localization is critical for proteolytic processing of human MBP (48, 49), and hsp70 has also been shown to be present in this compartment in a variety of cells (50).

Further studies will be required to provide definite evidence for the role of hsp in autoimmune responses, but based on the current findings it is tempting to hypothesize that overexpression of hsp70 resulting from a noxious environment during inflammatory processes can lead to enhanced Ag recognition.

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References


