In Vivo Cytotoxic T Lymphocyte Elicitation by Mycobacterial Heat Shock Protein 70 Fusion Proteins Maps to a Discrete Domain and Is CD4+ T Cell Independent

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Abstract

To gain insights into the mechanisms by which soluble heat shock protein (hsp) fusions can elicit CD8+ cytotoxic T lymphocytes (CTLs) against the fusion partner, mycobacterial (*Mycobacterium tuberculosis*) hsp70 was dissected to ascertain whether a particular hsp domain is necessary, and knockout mice were used to determine whether the fusion protein's immunogenicity is dependent on CD4+ T lymphocytes. We found that the ability to elicit CD8+ CTLs depends on a discrete 200–amino acid protein domain, indicating that the fusion protein's immunogenicity for CD8+ T cells does not require coupled chaperone function or peptide binding. Further, we found that ovalbumin (OVA).hsp70 fusion protein elicited anti-OVA CD8+ CTLs about equally well in CD4 knockout and wild-type C57BL/6 mice, and also when the hsp70 was of murine (self) origin. The ability of hsp70 fusion proteins to elicit CD4-independent CTL responses suggests that hsp70 fusion proteins may be useful for immunological prophylaxis and therapy against disease in CD4+ T cell–deficient individuals.

Key words: CD8+ • CD4+ • domain • knockout • vaccine

Introduction

There is now substantial evidence that heat shock proteins (hsps) isolated from tumors can be used as adjuvant-free antitumor vaccines in animals; hsp70 and the distantly related chaperones gp96 and calreticulin share this immunostimulatory activity (1–6). The fusion of large polypeptides (80–110 amino acids [aa] in length) to mycobacterial hsp70 (TBhsp70) creates potent immunogens that can elicit MHC class I–restricted, CD8+ CTL responses sufficient to mediate rejection of tumors expressing the fusion partner (7). The need for more effective immunological prophylaxis and therapy for cancer and infectious diseases caused by intracellular pathogens has spurred intense investigation of immunogens and immunization strategies aimed at eliciting effective CD8+ CTL responses. For example, CTL epitopes have been expressed in the context of Ty virus–like particles, or tailed with lipids (8). Antigenic peptides have been fused to toxins (9–11), cytokines (12, 13), and proteins that cross cell membranes, such as HIV-1 Tat (14), and hsp70 (7).

The means by which soluble hsp70 fusion proteins stimulate CD8+ CTL responses are unknown. Among the possible mechanisms are (a) strong hsp-specific CD4+ helper cell responses that enhance what might otherwise be a minimal response to the soluble proteins (15–18), and (b) the chaperone function of hsps that delivers the fusion protein to intracellular compartments of APCs for processing into short peptides and loading onto MHC class I (19, 20). We demonstrate here that hsp70 fusion proteins can elicit CD8+ CTLs in the absence of CD4+ T lymphocytes and that this function resides in a 200-aa segment of TBhsp70, indicating that chaperone activity is not required.

Materials and Methods

Expression Vectors.   All constructs used to produce OVA.hsp70 fusion proteins were made in the bacterial expression plasmid pKS11h (7). Fusion constructs, consisting of OVA fused to the NH$_2$ terminus of various segments of hsp70, were inserted downstream of the histidine tag sequence. A portion of OVA (aa 230–359) was amplified from pOV230 (21) by PCR using upstream primer oQH025 and the downstream primer oQH027 (the sequences of these and other PCR primers are listed at the end of Materials and Methods). The OVA expression vector pQH07

was constructed by subcloning OVA into the NdeI and NheI sites of pKS11h. Full-length TBhsp70 and four truncated TBhsp70 segments I (aa 1–166), II (aa 161–370), III (aa 360–517), and IV (aa 510–625), were amplified from plasmid pY3111/8 (gift of W. Wu, StressGen Biotechnologies, Victoria, Canada). The upstream primer for the full-length TBhsp70 and segment I is oQH001, and the downstream primers are oJR061 and oQH011, respectively. The upstream primers for TBhsp70 II, III, and IV are oQH012, oQH014, and oQH016, respectively; the downstream primers are oQH013, oQH015, and oJR061, respectively. The plasmids pQH06, pQH08, pQH09, pQH10, and pQH11, which express OVA fused to TBhsp70, TBhsp70 I, II, III, and IV, respectively, were constructed by subcloning the full-length and truncated TBhsp70 PCR products into the BamHI and EcoRI sites of pQH07 (at the COOH terminus of OVA). Murine hsp70.1 coding sequence (mhsp70) was amplified from plasmid pmhsp70.1 by PCR using the upstream primer oJR102 and the downstream primer oJR103. Plasmid pQH12, expressing OVA. mhsp70 fusion protein, was created by subcloning mhsp70 into the BamHI and EcoRI sites of pQH07. All plasmids were verified by sequencing in both directions with double-stranded DNA templates.

Recombinant Protein Purification. OVA, OVA.TBhsp70, OVA. TBhsp70 II, OVA.TBhsp70 III, and OVA.TBhsp70 IV were induced in *Escherichia coli* (BL21[DE3]pLysS) for 9 h at 25°C in the presence of 0.5–1 mM isopropyl thiogalactoside (IPTG), and were purified as soluble proteins. The mycobacterial segment I and murine hsp70 fusion proteins were induced in *E. coli* for 4 h at 37°C with 1 mM IPTG, purified from inclusion bodies, and then refolded as described previously (7, 16). All proteins were purified using nitrilo-triacetic acid Ni$^+$ column (Qiagen) and Hi-Trap® Q anion exchange chromatography (Amersham Pharmacia Biotech), as described previously (7, 16). Purity was assessed using 4–20% gradient SDS-PAGE gels stained with Coomassie blue (Bio-Rad). All proteins were dialyzed against PBS and sterile filtered at 0.2 μM. Protein concentrations were measured by Lowry assay (Bio-Rad) and expressed in molar terms to allow simple comparison of proteins of differing molecular weights.

Mice and Immunizations. 6–8-wk-old female C57BL/6, CD4$^{-/-}$, and β2-microglobulin (β2m)$^{-/-}$ mice were obtained from The Jackson Laboratory and Taconic Farms. Both knockout mice have C57BL/6 (H-2^b) genetic backgrounds. Groups of three or four mice were injected intraperitoneally with 120 pmol of recombinant protein in PBS; a second injection was performed subcutaneously 2 wk later. The mice were killed 10 d after the boost, and splenocytes within groups were pooled (7).

Cell Line. EG7-OVA cells were cultured as described previously (7).

CTL Assays. CTL assays were performed as described (7). Results shown are representative of experiments repeated two to five times.

The PCR primer sequences were as follows: for oQH025, 5′-GCAGTACTCATATGATCCTGGAGCTTCCATTTGCC-AGTGGGACAATG-3′; for oQH027, 5′-CTCCGACCTCAC-CTACGACGTTCGCAGAGACTTCTTAAAATTATCCGATG-CTAGACCTAGT-3′; for oQH001, 5′-ATAGTACTGGATC-CATGGCTCGTGCGGTCGGGATCGACCTCGGG-3′; for oJR061, 5′-GGAATTCCTATCTAGTCACTTGCCCTCCCG-GCCGTC-3′; for oQH011, 5′-GTCGACGAATTCATCATCA-GATCGCGCTCTTCTCGCCCTTGTCGAG-3′; for oQH012, 5′-GTCGACGGATCCATGGAGAAGGAGCAGCGAATCCT-GGTCTTCGACTTG-3′; for oQH014, 5′-GTCGACGGATCCA-TGGTGAAAGACGTTCTGCTGCTTGATGTTACCCCG-3′;

for oQH016, 5′-GTCGACGGATCCATGCGTAATCAAGC-CGAGACATTGGTCTACCAGACG-3′; for oQH013, 5′-GTC-GACGAATTCATCACGGGGTAACATCAAGCAGCAGAAC-GTCTTTCAC-3′; for oQH015, 5′-GTCGACGAATTCATCA-GACCAATGTCTCGGCTTGATTACGAACATCGGC-3′; for oJR102, 5′-TCTAGAGGATCCATGGCCAAGAACACGGC-GATC-3′; and for oJR103, 5′-TCTAGAGAATTCCTAATC-CACCTCCTCGATGGTGGGTCC-3′.

Results and Discussion

Our previous studies demonstrated that soluble, adjuvant-free TBhsp70 fusion proteins elicit substantial immune responses, including CD8$^+$ CTLs, in mice (7, 16). The basis for the effectiveness of hsp70 fusions is unclear, as most soluble proteins do not elicit significant CD8$^+$ T cell responses (for reviews, see references 22 and 23). Although there is evidence that the hsp moiety of TBhsp fusion proteins acts as an effective carrier in the classic sense, enhancing B cell responses to chemically conjugated pneumococcal polysaccharides (18) and malarial polypeptide (15), carriers are not known to stimulate CTL production. We thought it more likely that hsp70 fusion proteins provide hsp70-specific cognate CD4$^+$ T cell help to OVA-specific CD8$^+$ CTLs by activating shared professional APCs, as suggested by many and demonstrated recently (24–26).

We tested this cognate help hypothesis using CD4-deficient (knockout) mice (CD4$^{-/-}$). Wild-type C57BL/6, CD4$^{-/-}$, and β2m$^{-/-}$ mice were each immunized with OVA or OVA.TBhsp70 fusion protein. As expected, immunization of wild-type mice with OVA.TBhsp70, but not OVA, generated CTLs specific for the immunodominant epitope of OVA (SIINFEKL; Fig. 1 A). The same results were obtained when the CD4$^{-/-}$ mice were immunized with OVA.TBhsp70 (Fig. 1 B). β2m$^{-/-}$ mice, which have very few CD8$^+$ T cells, did not develop OVA-specific CTLs after immunization with OVA.TBhsp70 or with OVA alone (Fig. 1 C).

Previous efforts to determine whether CD4$^+$ T cell help is necessary for generation of CD8$^+$ CTLs have drawn differing conclusions. CD4 knockout mice exhibit a range of CD8$^+$ CTL responses: CD4-dependent, weakly dependent, or independent. CTL responses to minor histocompatibility antigens (25, 27) or to OVA loaded into spleen cells (28) are CD4 dependent. Some potent CD8$^+$ T cell immunogens, including viruses (29) such as lymphocytic choriomeningitis virus (30–33), ectromelia virus (34), and some influenza virus subtypes (35), as well as allogeneic cells (36), elicit strong CD8$^+$ T cell responses in wild-type and CD4$^{-/-}$ mice. The CD4 independence of the CTL response seen in Fig. 1 B does not exclude some role for CD4$^+$ help in normal mice immunized with hsp70 fusion proteins. Although it is possible that CD4$^-$/CD8$^-$ double-negative T cells provide weak residual help in CD4$^{-/-}$ mice, it is unlikely that such weak help is responsible for the anti-OVA CTLs seen in Fig. 1 B. The similarity of CD8$^+$ CTL responses to OVA.TBhsp70 in CD4$^{-/-}$ and wild-type mice suggests that hsp70 fusion proteins are relatively
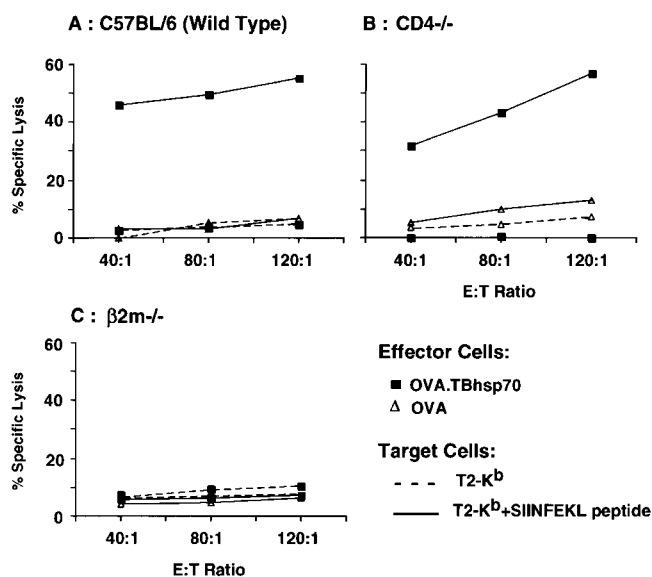
Figure 1. OVA-specific CTLs elicited by immunization with OVA.TBhsp70 fusion protein without adjuvant. The splenocytes from mice immunized with OVA (△) or OVA.TBhsp70 (■) were incubated with irradiated EG7-OVA cells for 6 d in the absence of added cytokines, and then used as effector cells (E) in a standard 4-h cytotoxicity assay. The ^{51}Cr-labeled target cells (T) were T2-K^b (dashed line) and T2-K^b pulsed with SIINFEKL peptide (solid line) at 33 μg/ml. (A) Splenocytes from wild-type C57BL/6 mice. (B) Splenocytes from CD4$^{-/-}$ mice. (C) Splenocytes from β2m$^{-/-}$ mice (this β2m$^{-/-}$ experiment was not repeated).

Figure 2. Murine hsp70 fusion protein elicits CTL responses in wild-type and CD4$^{-/-}$ mice. Soluble OVA.mhsp70 (●) was injected without adjuvant into C57BL/6 mice (A) and CD4$^{-/-}$ mice (B).

potent CD8$^+$ CTL immunogens. A similar result, showing that CD4$^+$ T cells are not required for the CD8$^+$ CTL response elicited by another mycobacterial heat shock fusion protein (hsp65 fused to a polypeptide containing an epitope for 2C CD8$^+$ T cells), has also been obtained using CD4$^{-/-}$ mice (Cho, B., personal communication). In addition, the ability of a nonhomologous hsp, gp96, to elicit tumor rejection requires CD4$^+$ T cells at tumor challenge, but not during priming with tumor-derived gp96 (2).

It has been proposed that the immunostimulatory effects of certain hsp fusion proteins may be due to the bacterial origin of the hsp moiety (20). We examined this possibility by making OVA.hsp70 fusion proteins with the murine homologue of TBhsp70 (37), here referred to as mhsp70. Immunization of wild-type C57BL/6 mice with OVA.mhsp70, but not OVA, elicited CTL responses equivalent to those generated by the TBhsp70 fusion protein (Fig. 2 A). The response to OVA.mhsp70 was also independent of CD4 (Fig. 2 B). Since a CD4$^+$ T cell response to self (murine) hsp70 is unlikely, the effectiveness of the murine hsp70 fusion protein is in accord with the more direct evidence for CD4 independence obtained using CD4$^{-/-}$ mice (see above).

The ability of hsp fusion proteins to elicit CTLs against the fusion partner may be a consequence of the hsp moieties' chaperone activity, assuming that this activity is preserved in the fusion protein. To investigate this issue, we divided TBhsp70 into four linear segments and fused OVA to the NH$_2$ terminus of each segment, creating OVA.TBhsp70s I–IV (Fig. 3 A). Each segment corresponds
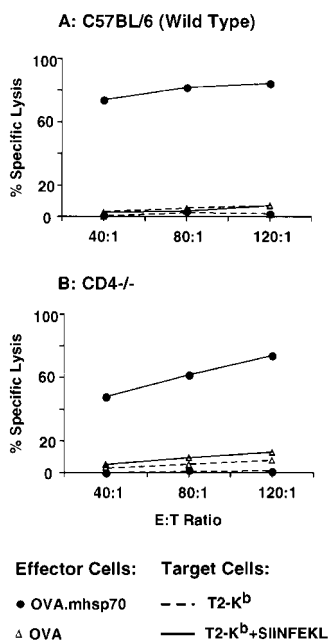
to a distinct structural domain of hsp70, as described by Flaherty et al. (38) and Zhu et al. (39). As shown in Fig. 3 A, the NH$_2$-terminal ATP-binding domain was divided into its two structural lobes: I (aa 1–166) and II (aa 161–370). The COOH-terminal peptide-binding domain was divided into a β sandwich domain, III (aa 360–517), and an α helical domain, IV (aa 510–625).

Six groups of three C57BL/6 mice were immunized with 120 pmol of OVA, OVA.TBhsp70, and OVA fused to segments I, II, III, and IV. CTL assays showed that splenocytes from mice immunized with OVA.TBhsp70 and OVA fused to segment II lysed T2-K^b cells in the presence, but not absence, of the OVA K^b epitope, SIINFEKL (Fig. 4). In contrast, cells from mice immunized with OVA and OVA fused to segments I, III, and IV were ineffective, even at an E/T ratio of 80:1. Levels of cytolysis obtained with splenocytes from mice immunized with OVA.TBhsp70 and OVA fused to segment II were indistinguishable (Fig. 4). These results show that half of the ATP-binding domain of TBhsp70 (aa 161–370) is sufficient to stimulate substantial production of anti-OVA CTL response in the absence of adjuvant.

Since it is highly unlikely that segment II retains chaperone activity, we conclude that the ability of the fusion proteins to elicit CD8$^+$ T cells does not depend on the hsp moieties' chaperone properties. How then can one account for the ability of these heat shock fusion proteins to act as CD8$^+$ CTL immunogens? Our data would support a model in which hsp70 bypasses the need for CD4$^+$ help by directly or indirectly activating or affecting the maturation state of APCs, such as dendritic cells, in a manner similar to some viruses (40). According to this model, hsp70 fusion proteins may activate a few CD8$^+$ T cells to release immunostimulatory cytokines in draining LNs. These cytokines may, in turn, provide the help required to upregulate expression of costimulatory molecules on APCs in the LN,

A: Hsp70 Domains
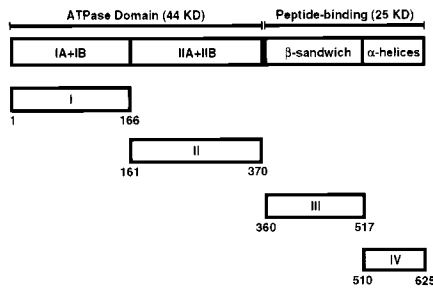
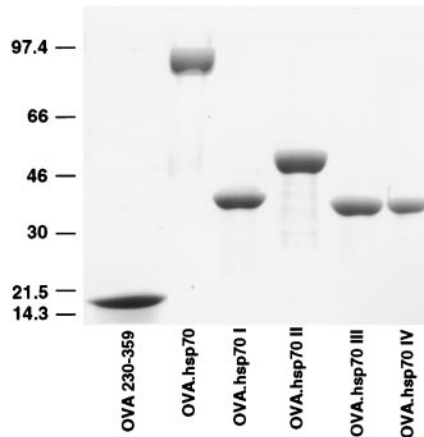## B: Purified OVA.TBhsp70 fusion proteins

Figure 3. Production of OVA.TBhsp70 fusion proteins using TBhsp70 segments. (A) Functional and structural domains of TBhsp70 based on crystal structures of the ATPase domain of bovine hsp70 (reference 38) and the peptide-binding domain of *E. coli* DnaK (reference 39). The full-length TBhsp70 was separated into four segments, I, II, III, and IV. The full-length TBhsp70 and each segment were fused to the COOH terminus of OVA to make OVA.TBhsp70 fusion proteins. The numbers beneath each segment refer to the aa positions in TBhsp70. (B) Purification of OVA.TBhsp70 fusion proteins. Purity was assessed using SDS-PAGE and visualized with Coomassie blue. Each lane was loaded with ~4 μg of protein. The numbers at left represent molecular weight markers (in kD).

leading to further CD8+ T cell activation (40). Recent studies demonstrate that exposure of macrophages to bacterial and human hsp60 (41, 42), murine hsp70, and gp96 (3, 43) increases expression of adhesion molecules and cytokines. We are currently examining expression of costimulatory molecules, adhesion molecules, and cytokines by APCs after exposure to fusion proteins made with full-length hsp70 or segment II.

Whatever the underlying mechanism, the ability of hsp70 fusion proteins to elicit CTL responses in the absence of CD4+ cells suggests that hsp70 may be a useful vehicle for the development of prophylaxis and therapy of HIV-1 and its opportunistic infections. Infection by HIV and its cousin simian immunodeficiency virus (SIV) can lead to a substantial reduction in CD4+ T cells, thereby crippling the host's immune response to HIV and other pathogens. This loss of CD4+ cells is thought to impair the development and maintenance of CD8+ CTL responses (44). Recent studies conclude that strong HIV-specific CTL responses are required to keep HIV-1 infection in check and to destroy HIV-infected cells (45–50). It will thus be of interest to determine whether hsp70 fusion constructs can elicit anti-SIV CTL responses in SIV-infected macaques having low CD4+ T cell counts, and if similar effects are observed in HIV-infected humans.
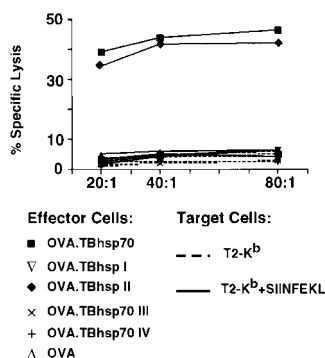

Figure 4. Examination of OVA-specific T cell responses in mice immunized with OVA fused to domains of TBhsp70. Splenocyte cultures from mice primed with OVA (△), OVA.TBhsp70 (■), OVA.TBhsp70 I (▽), II (◆), III (x), and IV (+) were used as effector cells in the cytotoxicity assay.

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